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Investigating novel approaches to the detection of virus neutralising antibodies to rabies and Rift Valley fever virus.

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BSc (Hons), MRes

Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy

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Abstract

Serosurveillance is a powerful tool fundamental to understanding infectious disease dynamics. The presence of virus neutralising antibody (VNAb) in sera is considered the best evidence of infection, or indeed vaccination, and the gold standard serological assay for their detection is the virus neutralisation test (VNT). However, VNTs are labour intensive, costly and time consuming. In addition, VNTs for the detection of antibodies to highly pathogenic viruses require the use of high containment facilities, restricting the application of these assays to the few laboratories with adequate facilities. As a result, robust serological data on such viruses are limited. In this thesis I develop novel VNTs for the detection of VNAb to two important, highly pathogenic, zoonotic viruses; rabies and Rift Valley fever virus (RVFV).

The pseudotype-based neutralisation test developed in this study allows for the detection of rabies VNAb without the requirement for high containment facilities. This assay was utilised to investigate the presence of rabies VNAb in animals from a variety of ecological settings. In this thesis I present evidence of natural rabies infection in both domestic dogs and lions from rabies endemic settings. The assay was further used to investigate the kinetics of VNAb response to rabies vaccination in a cohort of free-roaming dogs.

The RVFV neutralisation assay developed herein utilises a recombinant luciferase expressing RVFV, which allows for rapid, high-throughput serosurveillance of this important neglected pathogen. In this thesis I present evidence of RVFV infection in a variety of domestic and wildlife species from Northern Tanzania, in addition to the detection of low-level transmission of RVFV during inter-epidemic periods. Additionally, the investigation of a longitudinal cohort of domestic livestock also provided evidence of rapid waning of RVF VNAb following natural infection.

Collectively, the serological data presented in this thesis are consistent with existing data in the literature generated using the gold standard VNTs. Increasing the availability of serological assays will allow the generation of robust serological data, which are imperative to enhancing our understanding of the complex, multi-host ecology of these two viruses.

to Drew

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Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Abbreviations

ABLV	Australian bat lyssavirus
ACIP	Advisory Committee on Immunisation Practices
AIC	Akaike information criterion
ANDV	Andes virus
ARAV	Aravan virus
AUC	Area under curve
BBLV	Bokeloh bat virus
bp	Base pairs
BSL	Biosafety level
BUNV	Bunyamwera virus
BUNV-luc NT	Bunyamwera virus luciferase neutralisation test
C	Celsius
CFT	Complement fixation test
CI	Confidence interval
CPE	Cytopathic effect
CVS-11	Challenge virus standard-11
d	Day
df	Degrees of freedom
DI	Defective interfering
DNA	Deoxyribonucleic acid
DUVV	Duvenhage virus
EBLV	European bat lyssavirus
ELISA	Enzyme linked immunosorbent assay
FAVN	Fluorescent antibody virus neutralisation
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
h	Hours
HIV	Human immunodeficiency virus
HIV(luc) NT	Neutralisation test utilising luciferase expressing HIV pseudotypes bearing rabies glycoprotein
hpi	Hours post-infection
HRIG	Human rabies immunoglobulin

IEP	Inter-epidemic periods
IFA	Indirect fluorescent antibody test
Ig	Immunoglobulin
IKOV	Ikoma lyssavirus
IRKV	Irkut virus
IU	International Units
kb	Kilobase
kDa	Kilodalton
KHUV	Khujand virus
LAT	Latex agglutination test
LBV	Lagos bat virus
LLEBV	Lleida bat lyssavirus
m	Month
min	Minutes
MLV	Murine leukaemia virus
MOI	Multiplicity of infection
MOKV	Mokola virus
n	Number
NCA	Ngorongoro Conservation Area
OIE	World Organisation for Animal Health
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pNT	Pseudotype neutralisation test
PRNT	Plaque reduction neutralisation test
RdRP	RNA-dependent RNA polymerase
RFFIT	Rapid fluorescent focus inhibition test
rlu	Relative light units
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RRP	Rift replicon particles
RT-PCR	Reverse transcriptase polymerase chain reaction
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RVFV-luc NT	Rift Valley fever virus luciferase neutralisation test

rVSV-ΔG	Recombinant vesicular stomatitis virus in which the glycoprotein has been deleted
SAPO	Specified Animals Pathogens Order
SFSV	Sandfly fever Sicilian virus
SFTSV	Severe fever with thrombocytopenia virus
SHIBV	Shimoni bat virus
SNP	Serengeti National Park
TCID	Tissue Culture Infective Dose
TOSV	Toscana virus
tp	Time point
VNAb	Virus neutralising antibody
VNT	Virus neutralisation test
VSV	Vesicular stomatitis virus
WCBV	West Caucasian bat virus
WVI	Wildlife Vets International
y	Years

Chapter 1. Introduction

1.1 Serological diagnosis

1.1.1 Serology

At one time serology was the backbone of viral diagnosis, however, for many infectious diseases it is gradually being replaced by molecular techniques such as real-time polymerase chain reaction (PCR) and next generation sequencing. During the acute phase of infection, a diagnosis can only be achieved using serological methods if virus specific immunoglobulin (Ig) M is detected in serum, or if a four-fold rise in antibody titre is observed between acute and convalescent sera, in which case diagnosis is retrospective. Additionally, the immunological response to many acute viral infections occurs after the development of clinical symptoms which limits the use of serology in clinical management of cases in the early stage of infection. Nonetheless, serology remains a core component of the routine diagnostic virology laboratory. Serological tests can provide important information such as a) diagnosing acute infection by detecting IgM antibodies; b) detecting antibodies which always indicate current infection (for example in HIV); c) the identification of patients with past exposure who may be at risk of recurrent infection; d) determining the immune status following vaccination or natural exposure and e) identifying risk factors of infection through retrospective analysis.

Serology also remains a powerful tool for investigating virus ecology and infection dynamics. Antibodies often last considerably longer than viral nucleic acid and are therefore a more sensitive indicator of infection prevalence in a population compared to molecular techniques. Serosurveillance remains a critical component in control and elimination strategies of many important viral animal diseases, as shown by the successful eradication of rinderpest (OIE, 2011) and current campaigns to control peste de petits ruminants in Africa (OIE and FAO, 2015). The serum biobanks that exist across Africa have great potential for

serological investigations of other diseases, but have rarely been used for secondary analyses. In wildlife, where opportunities for sample collection are more limited than for human or domestic animals, serology is often the only available tool for inference and modelling of infectious disease dynamics (Gilbert et al., 2013). However, data produced by serological assays with poor sensitivity or specificity, or those affected by cross-reactivity or unclear cut-off thresholds complicate the interpretation of serological data (Viana et al., 2016). Thus sensitive, specific and robust serological assays are required to produce informative data regarding the prevalence and transmission dynamics of viruses.

In addition to the innate immune system which is activated upon infection, specific acquired immunity occurs in response to viral antigens. The acquired immune response refers to the production of antibodies specific to the inducing antigen. Antibody secreting plasma cells are capable of secreting five classes of immunoglobulins; IgA, IgM, IgG, IgD and IgE. Three of these isotypes, IgA, IgM and IgG, are capable of virus neutralisation (Klimpel, 1996), which allows antibodies to block viral infection. Enveloped viruses can be neutralised by preventing the attachment of virus to cell surface receptors, by blocking penetration of the virus into the cell after attachment or by preventing uncoating of the virus within the cell. Virus neutralising antibodies (VNAb) are raised to viral glycoproteins and for many viral infections the presence of VNAb is one of the best correlates of protection from infection (Klasse, 2014).

1.1.2 Serological assays

Several assays are available for detecting virus specific antibodies, including the complement fixation test (CFT), indirect fluorescent antibody test (IFA), enzyme linked immunosorbent assay (ELISA) and the virus neutralisation test (VNT). In the CFT antibodies are detected based on the reaction of complement with antigen-antibody complexes. Briefly, test sera are heat treated and mixed with a fixed concentration of complement, antigen is then added and the test is incubated. The serum-antigen complex is then added to sensitised red blood cells, which lyse in the absence of antibody and remain intact if antibody is present in the test serum. To detect antibodies using the IFA test, antigen is coated onto a microscope slide and test sera are added. A fluorescently labelled secondary antibody, specific to the host species being tested, is then added to

the slide, if antibody is present fluorescence is observed under a fluorescence microscope. The ELISA employs a similar principle to the IFA, however a 96-well format is most commonly used and the secondary antibodies are coupled to an enzyme instead of a fluorophore, allowing a colour change to be determined by measuring the optical density. Depending on the ELISA platform used, either the presence or absence of colour indicates the presence of antibodies. In both the IFA and ELISA, IgM or IgG can be detected using isotype-specific secondary antibodies.

The antibodies detected by the CFT, IFA and ELISA may be either neutralising or non-neutralising and the tests cannot differentiate between the two. The VNT is the only assay available to measure the presence of VNAb and is generally considered the gold standard assay for antibody detection (Monath et al., 2012). The test protocol involves mixing dilutions of test sera with a fixed amount of virus and incubating to allow VNAb to neutralise the virus. The serum/virus mix is then added to a monolayer of permissive cells and incubated for a period sufficient for the virus to infect cells and produce a cytopathic effect (CPE). Cells are then viewed under the microscope, the presence of VNAb is determined by the absence of CPE, as VNAb in the serum have successfully neutralised the virus. For viruses which do not produce CPE, labelled antibodies directed against the virus can be used to detect virus in the cell culture.

Although the VNT is considered the gold standard for antibody detection, the assay requires the use of cell culture, live virus and involves incubation for a number of days before a result is available. Additionally, the test is laborious and few samples can be investigated simultaneously, as each serum dilution requires testing in individual wells, and tests are often performed in 6 or 12 well plates (Mather et al., 2013). Interpretation of the VNT is also subjective, as wells are scored for the presence or absence of CPE (or plaques) and results can differ from operator to operator. Furthermore, although the VNT is considered the gold standard, false positives and false negatives still occur, as is the case in all serological assays. False positive results in the VNT may be due to the presence of non-specific inhibitors in serum, the detection of cross-neutralising antibodies or components in the serum causing cytotoxicity. False negative results may occur in the VNT if low levels of antibody are present in the serum. A major limitation of the VNT is the use of live virus, if the virus requires high

containment facilities the VNT must be performed in such facilities. The majority of viruses which require handling at high containment circulate in regions of the world where such facilities are seldom available and this can hamper serosurveillance efforts for such viruses.

1.1.3 Viral pseudotypes

More recently VNTs have been described in which live virus is substituted with viral pseudotypes. A pseudotyped virus is a hybrid virus particle consisting of the nucleocapsid core of one virus and the glycoprotein of a heterologous virus (Temperton et al., 2015). Pseudotypes are used widely for gene transfer, where genes of interest are delivered into cells by utilising the glycoprotein of a virus with a broad cell tropism such as vesicular stomatitis virus (VSV). Among the most commonly used viral pseudotypes for gene therapy are those based on retroviruses (Sanders, 2002). The use of pseudotypes as surrogate viruses in VNTs is also becoming increasingly popular (Bentley et al., 2015), in such cases the 'gene of interest' is a reporter gene, such as green fluorescent protein (GFP) or luciferase, with reporter gene expression indicating cellular entry. Retroviral pseudotypes are produced by transfecting plasmids into producer cells (often 293T). Transfected plasmids include 1) a packaging plasmid 2) a transfer vector containing the reporter gene and 3) a plasmid encoding the glycoprotein gene (Figure 1.1). The packaging plasmid contains gag and pol genes which produce the core viral proteins. The transfer plasmid contains the modified retroviral genome encoding the reporter gene which is flanked by the viral long terminal repeats, the intact packaging signal allows incorporation of the retroviral genome into the pseudotype. The glycoprotein expression plasmid allows the viral glycoprotein to be expressed within the producer cells. Transfected genes are transcribed and translated within the producer cells and the glycoprotein is expressed on the cell surface. The retroviral genome carrying the reporter gene is incorporated into the pseudotype via the packaging signal, and the nucleocapsid core is targeted to the plasma membrane where the pseudotype buds (Temperton et al., 2015). Pseudotyped viruses are released into the supernatant of the producer cells and are then able to be used for transduction of target cells, where they can be used for applications such as investigating cellular tropism, identifying antiviral compounds or used in neutralisation assays.

The glycoproteins of the pseudotype mediate cellular attachment via cell surface receptors on target cells. The pseudotype is internalised and the retroviral construct bearing the reporter gene is integrated into the host cell DNA and expressed. Therefore, the level of reporter gene expression correlates with the level of transduction of pseudotype particles (Temperton et al., 2015). As the defective retroviral genome packaged into the pseudotype carries only the reporter gene, but no viral genes, no viral proteins are expressed in the target cells. Hence the pseudotypes are infectious but replication defective, which is their greatest advantage when we consider their use in neutralisation tests. The glycoproteins of highly pathogenic viruses such as rabies, Ebola, Japanese encephalitis virus and highly pathogenic strains of influenza A have been used to pseudotype retroviruses (Wright et al., 2008, Wool-Lewis and Bates, 1998, Lee et al., 2014, Temperton et al., 2007) resulting in pseudotypes that can be used to study these highly pathogenic viruses at biosafety level (BSL) 2.

In addition to retroviral pseudotypes, rhabdoviral pseudotypes can also be produced using a modified VSV in which the glycoprotein has been deleted and replaced with a reporter gene. Due to the mechanism of VSV budding, which allows budding to occur in the absence of glycoprotein, this recombinant VSV can therefore be used to produce pseudotypes using the glycoprotein of heterologous viruses (Whitt, 2010).

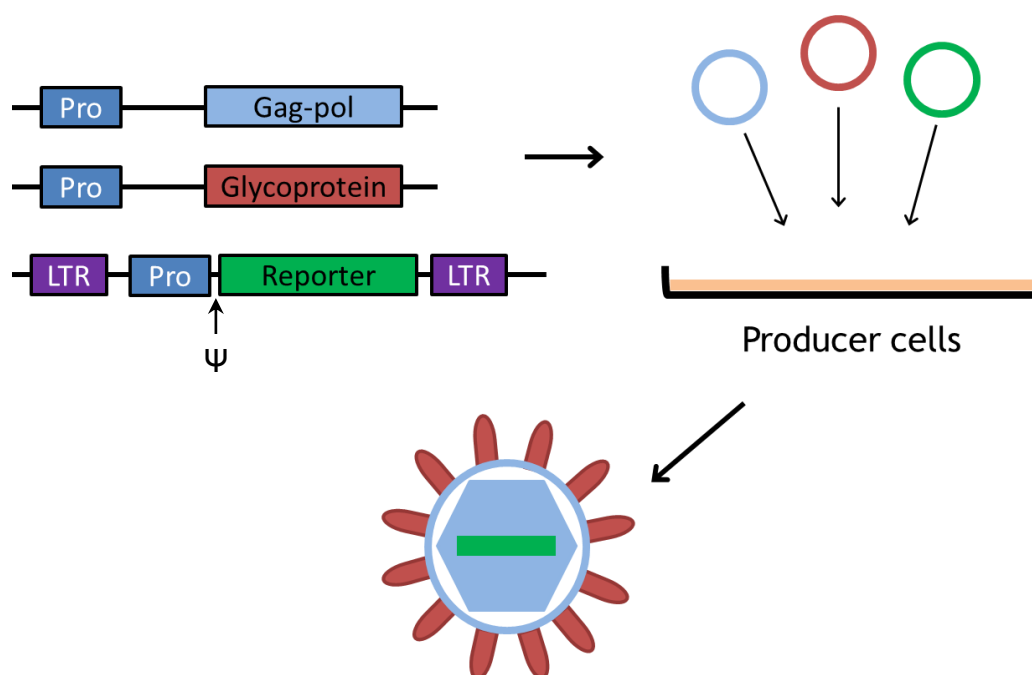


Figure 1.1 Schematic representation of retroviral pseudotype production. Schematic diagram of a three-plasmid transfection system for producing retroviral pseudotypes. Transfected plasmids comprise of a retroviral packaging plasmid containing the viral gag-pol, a plasmid encoding the glycoprotein of interest and a transfer vector expressing the reporter gene containing the packaging signal and flanked by long terminal repeats (LTR). The three plasmids are simultaneously transfected into producer cells (usually 293T cells) and 48 h later supernatants containing retroviral pseudotypes are harvested. PRO=promoter. Figure adapted from Temperton et al. (2015).

1.1.4 Pseudotype-based neutralisation tests

As neutralising antibodies are elicited to viral glycoproteins, pseudotypes can be used to replace live virus in the VNT. The pseudotype neutralisation test (pNT) follows the same protocol as previously described for the VNT, however serum is mixed with pseudotypes in place of virus and instead of observing CPE, reporter gene expression is detected (Figure 1.2). Neutralisation is measured by an absence (or decrease) in reporter gene expression in the target cell line, as the

pseudotypes have been neutralised by VNAb in the sera before they have entered the cells and hence no (or less) reporter gene is expressed in the cells.

The main advantage of the pNT over the conventional VNT is that pseudotypes of highly pathogenic viruses can be used in BSL-2, hence neutralisation assays for such viruses can be performed outside of high containment, vastly increasing the number of laboratories capable of performing such assays. Further advantages of the pNT include the use of the reporter gene to detect pseudotype transduction. Using reporters such as luciferase removes the subjective nature of assessing whether cells are displaying CPE, as luciferase expression is measured as an absolute number. Although luciferase reporter genes have been termed the gold standard for pNTs (Mather et al., 2013), high costs hamper their use in resource limited laboratories. The flexibility of pseudotype production allows for various reporter genes to be included, based on the preference of individual laboratories. Pseudotypes bearing the *lacZ* reporter gene may be most suitable for use in the pNT in resource limited settings as this reporter system is 30 times less expensive than the luciferase equivalent (Wright et al., 2009). In addition, the use of reporter genes in pseudotypes lends itself to the development of high-throughput platforms for VNAb detection.

A further advantage of the pNT is that significantly smaller volumes of sera are required in comparison to the conventional VNT, typically requiring a 5-25 fold reduction in serum volume (Temperton et al., 2015). Wright et al. (2010) have also described a multiplex pNT in which different reporters are utilised for pseudotypes of different viruses and both are simultaneously detected in the same well. This demonstrates the potential to detect VNAb to multiple viruses using an already decreased serum volume, a strategy which would be particularly beneficial when screening for VNAb in wildlife species in which often little serum is available.

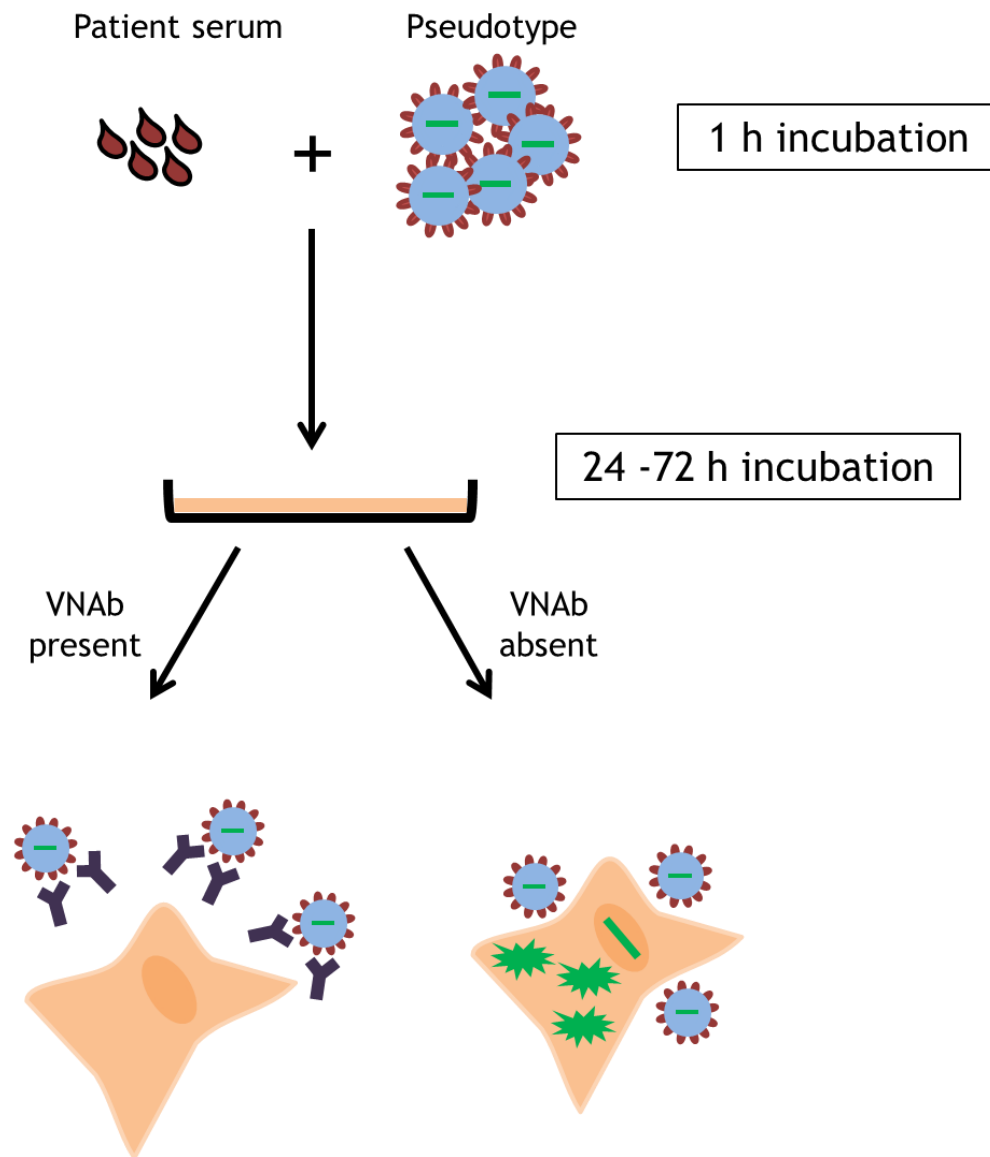


Figure 1.2 Schematic representation of the pseudotype neutralisation test. In the pseudotype neutralisation test, test sera is mixed with a fixed concentration of viral pseudotype. The serum/pseudotype mix is incubated for 1 h after which it is plated onto target cells and further incubated. If virus neutralising antibodies (VNAb) are present in the test sera, antibodies neutralise the pseudotypes and no reporter gene expression is observed in the target cells. In the absence of VNAb, pseudotypes transduce the target cells and expression of the reporter gene is observed.

Numerous studies have utilised pNTs for the detection of VNAb to a wide variety of viruses (Table 1.1). Previous studies have demonstrated that pNTs are as sensitive as their conventional VNT counterparts, and some have even demonstrated increased sensitivities compared to VNT (Temperton et al., 2007, Logan et al., 2016, Wright et al., 2008, Fukushi et al., 2006). The specificities of the pNTs described in the literature are also high, as the glycoprotein is the only protein present on the pseudotype membrane it can be inferred that any neutralisation demonstrated in the pNT is specifically due to interactions with the viral glycoprotein.

In this thesis I aim to utilise pseudotyped viruses to develop pNTs to two high containment viruses, rabies and Rift Valley fever virus (RVFV). Improving the availability of these assays will allow further data generation to advance our understanding of the ecology of these two important zoonotic viruses. However, RVFV pseudotypes were not produced to a high enough titre to allow for large scale screening of sera therefore other novel alternatives to the conventional neutralisation test were developed.

Viral species pseudotyped	Core vector system	Reporter gene	Reference
Rabies virus, Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssavirus types 1 and 2	HIV	Firefly luciferase	(Wright et al., 2008, Wright et al., 2009)
Influenza A virus	HIV/MLV	Firefly luciferase/GFP	(Temperton et al., 2007, Labrosse et al., 2010)
SARS coronavirus	MLV/VSV	GFP	(Temperton et al., 2005, Fukushi et al., 2006)
Feline immunodeficiency virus	HIV	Firefly luciferase	(Samman et al., 2010)
Canine distemper virus	VSV	Firefly luciferase	(Logan et al., 2016)
Nipah virus	VSV	GFP	(Kaku et al., 2009)
Severe fever with thrombocytopenia virus	VSV	Firefly luciferase	(Hofmann et al., 2013)

Ebola virus	HIV	Firefly luciferase	(Shedlock et al., 2010)
Japanese encephalitis virus	MLV	β -galactosidase	(Lee et al., 2014)
Rift Valley fever virus	VSV	Firefly luciferase	(Bukbuk et al., 2014)
Hantaan virus	VSV	<i>Renilla</i> luciferase/GFP	(Higa et al., 2012, Ogino et al., 2003)
Puumala virus	VSV	<i>Renilla</i> luciferase	(Higa et al., 2012)
Seoul virus	VSV	GFP	(Ogino et al., 2003)
Hepatitis C virus	HIV/MLV	Firefly luciferase/GFP	(Logvinoff et al., 2004, Bartosch et al., 2003)
Human immunodeficiency virus type 1	HIV	Firefly luciferase	(Simek et al., 2009)
Chikungunya virus	HIV	Firefly luciferase	(Kishishita et al., 2013)

Table 1.1 Neutralisation tests in which viral pseudotypes have been utilised as surrogates for live virus. Examples of viral pseudotypes which have been utilised in pNTs for the detection of virus specific VNAb.

1.2 Rabies virus

1.2.1 Rabies virus genome

Rabies is one of the oldest and most feared zoonotic diseases (Muller et al., 2014), and unsurprisingly as the disease proves fatal in very almost every case once symptoms occur. Rabies virus is the prototype of the *Lyssavirus* genus, family *Rhabdoviridae* (King et al., 2012). The virus has a single stranded, negative sense, unsegmented RNA genome which is approximately 12 kb in size and encodes five structural proteins; the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA-dependent RNA polymerase (RdRP) (L) (Jackson and Wunner, 2007) (Figure 1.3). Each gene is separated by non-coding intergenic sequences. The intergenic sequence between the G and L genes is unusually long (~423 bp) and is unique to members of the *Lyssavirus* genus (Tordo et al., 1986). Cellular entry is mediated by the viral glycoprotein, and although several putative rabies receptors have been identified, the definitive rabies receptor(s) have yet to be fully elucidated (Lafon, 2005).

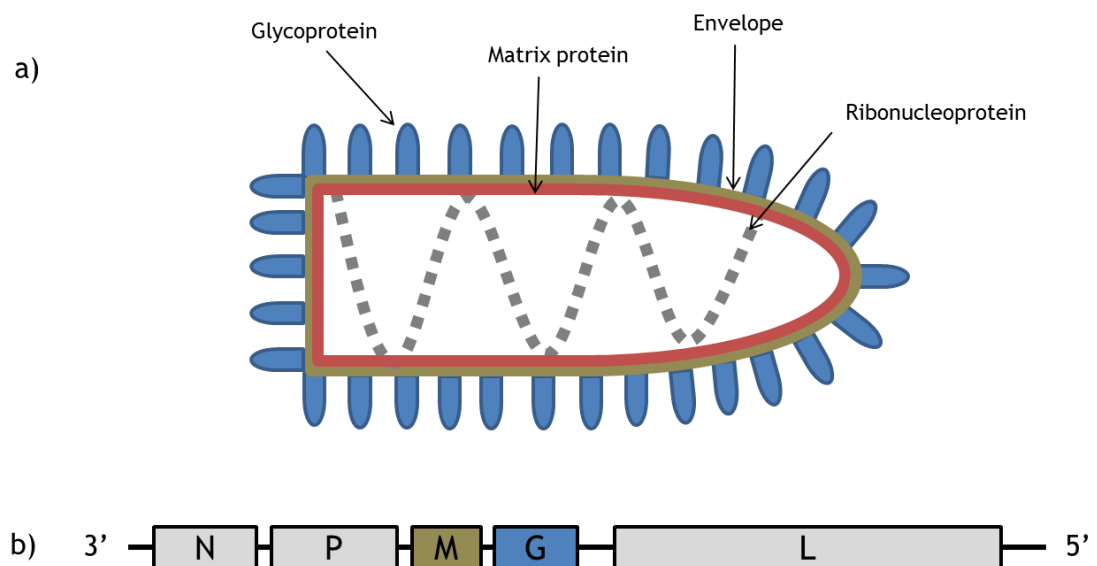


Figure 1.3 Schematic diagrams of rabies virus virion and the viral genome. Schematic representation of a) the rabies virus virion and b) the viral genome which encodes the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA-dependent RNA polymerase (L).

1.2.2 Host species

All mammals are susceptible to rabies virus infection, however only a limited number of host species act as reservoirs of the virus. Rabies virus demonstrates distinct phylogeographical patterns, with particular reservoir hosts associated with particular regions and viral strains. Domestic dogs are the principal host reservoir for rabies in Asia and Africa (Cleaveland et al., 2006). In much of Western Europe canine rabies has been eliminated, yet rabies still persists in East and South-East Europe where it is maintained in wildlife reservoirs (Freuling et al., 2012). Bats maintain rabies virus across the Americas with canine rabies co-circulating in Latin America and wildlife rabies maintained in North America by mesocarnivores. Throughout the world, distinct lineages of rabies virus are maintained in various carnivore reservoirs, which include raccoon dogs, skunks, coyotes, mongooses, raccoons and grey, red and arctic foxes (Müller et al., 2015). Interestingly, whilst rabies in carnivores is maintained globally, rabies virus is only observed in bats in the Americas and it appears Old World bats are not infected with rabies virus. They are however reservoir hosts of other lyssavirus species.

Although canine rabies has been eliminated in most developed countries, it is still the cause of around 59,000 human deaths globally each year (Hampson et al., 2015). Domestic dog vaccination campaigns are key to controlling canine rabies in endemic settings. Dog vaccination schedules suggest a single dose of vaccine followed by boosters every 1-3 years, depending on the duration of immunity specified by the vaccine manufacturer (Day et al., 2010). Attempts to control rabies in wildlife (excluding bats) rely upon oral vaccination programmes utilising vaccine-laden baits, which have resulted in elimination of rabies in many countries in Western Europe (Rupprecht et al., 2004).

1.2.3 Rabies virus infection

Rabies virus can be transmitted from reservoir host species to dead end hosts and such spillover infections occur in both humans and animals. The vast majority of human rabies infections occur via the bite of a rabid animal, as the virus is present in large quantities in the saliva (Fekadu et al., 1982). However, direct contact of infected bodily fluids with mucous membranes, such as by licks or scratches, are an additional route of natural infection. Rare cases of rabies

transmission through solid organ (Srinivasan et al., 2005) and corneal transplant (Gode and Bhide, 1988) have also been reported. Airborne transmission of rabies virus, due to exposure to bats in caves and also through laboratory associated infections, have been reported in humans (Johnson et al., 2006).

Initial clinical symptoms in humans in the prodromal phase may include fever, loss of appetite and pain at the site of infection. Two clinical forms of rabies can occur in humans; encephalitic or paralytic (Fooks et al., 2014), however overlap of clinical symptoms is often observed (Deitzgen and Kuzmin, 2012). The encephalitic form often presents with agitation, insomnia and hallucinations in addition to the less commonly observed features of hydrophobia, aerophobia and photophobia. In paralytic rabies muscle weakness occurs early and agitation and anxiety are mild or absent. In both forms of rabies, patients become comatose and die, due to respiratory failure or cardiac arrest, typically within 1-10 days post symptoms (Deitzgen and Kuzmin, 2012).

1.2.4 Treatment and vaccination

Although rabies is almost always fatal once clinical symptoms present, the disease is entirely preventable through vaccination in both humans and animals. Vaccination in humans is recommended for anyone at risk of infection, namely veterinarians, bat handlers, laboratory workers and those travelling to rabies endemic areas. Due to the serious consequences of infection and the possibility of waning antibody titres, a course of post-exposure vaccination is also recommended after a potential exposure, even for patients who have previously been vaccinated. Patients who receive a high risk rabies exposure and have not been prophylactically vaccinated, additionally receive human rabies immunoglobulin (HRIG) which is administered at the exposure site as soon as possible (PHE, 2013). The use of HRIG aims to neutralise the virus at the inoculation site, reducing the viral load and allowing time for the host's adaptive immune response to develop (Wilde et al., 2002).

Neutralising antibodies, raised against the viral glycoprotein, play a prominent role in the immune defence against rabies virus and the presence of VNAb confer protection against infection (Jackson and Wunner, 2007). During infection antibodies are also raised against the viral nucleoprotein, these antibodies are not neutralising but they have been shown to protect experimentally infected

mice in the absence of VNAb (Lodmell et al., 1993), however the exact mechanisms that infer such protection remain unclear.

1.2.5 Rabies diagnosis

Clinical symptoms and a history of potential rabies exposure are often sufficient for a clinical diagnosis of rabies infection, however in paralytic rabies clinical symptoms are often indistinguishable from those of other neurological conditions (Mani and Madhusudana, 2013). Furthermore, potential rabies exposures are often lacking from a patient's clinical history (Despond et al., 2002). The gold standard diagnostic assay for rabies infection is the fluorescent antibody test which detects viral antigen in tissue samples using immunofluorescence (OIE, 2012a). Other diagnostic tests include rabies virus isolation, antigen detection by ELISA and detection of viral RNA by molecular techniques.

Serology is rarely used for rabies diagnosis as seroconversion occurs late in disease and the negative predictive value of rabies antibody detection is low, as VNAb are often undetectable even in the terminal stages of disease (Mani et al., 2014). Additionally, if patients have received rabies vaccination or HRIG, antibody detection cannot be used diagnostically. However, serological testing is useful for demonstrating seroconversion following vaccination and for rabies epidemiological studies. The gold standard measure of seroconversion is the detection of rabies-specific VNAb by VNT. The World Organisation for Animal Health (OIE) prescribed VNTs are the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody virus neutralisation (FAVN) test.

The RFFIT is performed by mixing dilutions of test sera and the challenge virus standard-11 (CVS-11) strain of rabies virus in tissue culture chamber slides which are then incubated at 35°C in a humid incubator for 90 min. Mouse neuroblastoma cells or baby hamster kidney cells (BHK-21) are then added to the wells and incubated for a further 20 h. Media is removed and cells are washed in PBS, fixed in acetone and dried, after which FITC-conjugated anti-rabies serum is added to the slide and incubated for 30 min. Wells are then observed under fluorescence microscopy and the number of fluorescent cells are manually counted (Smith et al., 1973). The FAVN is a modified version of the RFFIT which retains a similar protocol, although requires 48 h incubation. The advantage of the FAVN over the RFFIT is that the read out of the FAVN is absolute, simply the

presence or absence of fluorescence, and is therefore considered less subjective than the RFFIT (Cliquet et al., 1998). Results of the RFFIT and FAVN can be expressed either as a serum dilution titre or in International Units (IU). For expression of titres in IU an international standard reference serum of known concentration is tested in parallel with test sera and antibody titres are derived from comparison with the reference standard.

Both the RFFIT and the FAVN utilise live virus and therefore must be performed within high containment facilities. Rabies virus is considered a Group 4 pathogen under the Specified Animals Pathogens Order (SAPO) and requires enhanced containment conditions for handling virus (DEFRA, 2015). These assays are time consuming and require highly trained individuals to perform and interpret. As a result, very few laboratories are able to perform these assays and their use is particularly hampered in developing countries, those that are most often rabies endemic. Although it is the gold standard, the RFFIT has been shown to be sensitive to cytotoxicity when poor quality sera are tested (Ma et al., 2012) and false positives can occur due to nonspecific inhibitors in sera (Mani and Madhusudana, 2013). Furthermore, interpretation of both the RFFIT and FAVN are subjective.

ELISAs are a simple, cheap, rapid and safe alternative to the VNT, no high containment facilities are required and this platform is much better suited to high-throughput testing. However, some studies have demonstrated poor correlation between the ELISA and VNT, especially when samples contain low concentrations of rabies antibody (Welch et al., 2009, Cliquet et al., 2004). It should also be noted that ELISAs are not functional assays, measuring antibody binding, not neutralisation, and therefore cannot be used to determine protection.

1.2.6 Alternatives to conventional virus neutralisation tests

Wright et al. (2008) described a rabies VNT utilising retroviral pseudotypes in place of live rabies virus. In this study they successfully produced pseudotypes using an HIV (lentivirus) core pseudotyped with rabies virus glycoprotein which expressed a luciferase reporter gene and an MLV (gammaretrovirus) rabies pseudotype expressing GFP. The lentiviral pseudotypes were used to measure rabies VNAb in human, canine and feline sera and the pNT titres were highly

correlated with VNAb titres obtained using the gold standard FAVN. These replication incompetent particles allow VNAb measurement without the high containment facilities required to handle live rabies virus, greatly improving the availability and utility of this assay. In addition, the study reported 100% specificity and was as sensitive at detecting VNAb positive samples as the FAVN.

Additional alternative assays for measuring rabies VNAb have also been described. Latex agglutination tests (LAT) use antigen coated latex beads incubated with serum to detect antibodies to rabies virus, in which agglutination signifies the presence of antibodies. Original tests used whole virus to coat the latex beads (Perrin et al., 1988), however to detect only glycoprotein-specific antibodies, methods utilising beads coated with only the viral glycoprotein have since been described (Jemima et al., 2014). The test is very simple and cheap to perform therefore large scale serosurveillance can be performed easily. Although these tests appear to be specific, the sensitivity is poor when low titres of antibody are present in test sera (Madhusudana and Saraswati, 2003, Jemima et al., 2014). This assay measures antibodies to the glycoprotein, which in addition to VNAb may also include non-neutralising antibodies, therefore the results of this assay cannot be used to infer protection. Additionally, measurements from LATs are only qualitative and cannot be used to determine specific titres of rabies VNAb in sera.

Rabies VNTs that utilise recombinant rabies virus expressing GFP, produced by reverse genetics techniques, have also been described (Khawplod et al., 2005, Xue et al., 2014, Tang et al., 2015). These tests demonstrate high sensitivity and specificity when compared with the RFFIT or FAVN. These tests are easier to interpret than conventional VNTs as GFP fluorescence is easily identified and post-assay processing is reduced as antibody staining is not required. However, these recombinant viruses are still infectious and replication competent, therefore still require handling in the high containment facilities associated with the RFFIT and FAVN.

The majority of rabies VNTs are used to determine whether or not a sufficient immune response has been mounted post vaccination and only a minority of testing occurs diagnostically to provide evidence of infection. VNTs are also performed for serosurveillance of lyssavirus antibodies in bats and rabies VNTs

are also used, although rarely, for rabies serosurveillance in terrestrial mammals. Non-fatal rabies infection is an accepted outcome of rabies infection in bats but such infection in other species challenges the theory that rabies infection is always fatal. There have been documented cases of recovery from rabies infection in animals during clinical trials and there have also been reports of rabies antibodies in healthy animals, suggesting non-fatal rabies infections in nature (Table 1.2). Potential explanations for such non-fatal infections include receiving a low infectious dose, spontaneous viral clearance or the presence of defective interfering (DI) particles. The presence of DI rabies virus particles have been demonstrated *in vivo* and *in vitro*, such particles are produced alongside functional virions during viral replication but are themselves non-infectious (Baer, 1991). The presence of DI particles can interfere with replication of non-defective rabies virions and such a phenomenon has been shown to actually protect experimentally infected animals from disease (Wiktor et al., 1977). The role of DI rabies virus particles in natural infection has not been studied but is a theoretical explanation for the presence of rabies VNAb in unvaccinated animals.

Exposure	Testing method	Species	Comments	Reference
Experimental infection	RFFIT	Ferret	One ferret recovered from rabies virus infection, demonstrating VNAb in serum and CNS	(Hamir et al., 2011)
Experimental infection	FAVN	Dog	An unvaccinated control dog survived rabies challenge, demonstrating VNAb at a titre of 0.22 IU/ml in serum	(Darkaoui et al., 2016)
Natural infection	RFFIT	Cattle	42/350 unvaccinated cattle from Guatemala had rabies VNAb titres of ≥ 0.1 IU/ml	(Gilbert et al., 2015)
Natural infection	RFFIT/IFA	Humans	6/63 humans from bat exposed communities had rabies VNAb with no history of previous vaccination	(Gilbert et al., 2012)
Natural infection	RFFIT	Humans	High VNAb titre in a fox trapper in Alaska with no previous history of rabies vaccination	(Follmann et al., 1994)
Natural infection	RFFIT	Dogs	115/233 unvaccinated dogs from a rabies endemic area of Tanzania demonstrated rabies VNAb	(Cleaveland et al., 1999)

Natural infection	FAVN	Dogs	24/123 unvaccinated dogs from a rabies endemic area of Tanzania demonstrated rabies VNAb	(McNabb, 2008)
Natural infection	FAVN	Dogs	VNAb in unvaccinated dogs from rabies endemic areas of South Africa and Indonesia (n=20)	(Morters et al., 2014a)
Natural infection	RFFIT	Mongoose	502/1675 of mongooses tested had rabies VNAb at serum dilutions of $\geq 1:5$	(Everard et al., 1981)
Natural infection	RFFIT	Jackal	3/16 unvaccinated animals had rabies VNAb titres at serum dilutions of $\geq 1:50$	(Mebatsion et al., 1992)
Natural infection	RFFIT	African wild dogs	3/12 unvaccinated animals had rabies VNAb titres	(Gascoyne et al., 1993)

Table 1.2 Studies in which rabies virus neutralising antibodies have been demonstrated in the absence of clinical disease or after recovery from rabies infection. Examples of studies demonstrating rabies VNAb in humans and animals in the absence of, or recovery from, clinical disease. Titres of VNAb recorded in the studies were ≥ 0.5 IU/ml unless otherwise stated. RFFIT=rapid fluorescent focus inhibition test, FAVN=fluorescent antibody virus neutralisation test, IFA=indirect fluorescent antibody test.

1.3 Non-rabies lyssaviruses

Rabies virus is the prototype virus of the *Lyssavirus* genus, of which 14 distinct species are recognised (King et al., 2012), with a further potential species (Lleida bat lyssavirus (LLEBV)) reported in 2013 (Aréchiga Ceballos et al., 2013) (Table 1.3). The majority of these lyssaviruses have been reported in bats; however Mokola virus (MOKV) and Ikoma lyssavirus (IKOV) have only been isolated from terrestrial mammals. Mokola virus was first identified in shrews (Shope et al., 1970) and has been further isolated from cats, dogs and a mouse (Sabeta et al., 2007). Ikoma lyssavirus has been identified only in one African civet from within the Serengeti National Park (Marston et al., 2012), and although this is likely to signify a spillover infection from a bat reservoir host, no evidence of IKOV infection in bats has been detected to date (Horton et al., 2014). All lyssaviruses have the same genomic structure as rabies virus (Delmas et al., 2008) and it has been proposed that lyssaviruses evolved in bats long before spilling over into terrestrial mammals (Badrane et al., 2001, Streicker et al., 2010). As with rabies virus the phylogeography of lyssaviruses is distinct, with specific lyssaviruses circulating in particular regions (Table 1.3). To date, spillover infections of non-rabies lyssaviruses caused by MOKV, Duvenhage virus (DUVV), European bat lyssavirus (EBLV) types 1 and 2, Australian bat lyssavirus (ABLV) and Irkut virus (IRKV) have been reported in humans (Evans et al., 2012). Transmission routes are identical to rabies virus and the clinical picture is indistinguishable from that of rabies virus infection. Human cases of non-rabies lyssavirus infection however are incredibly rare, only around 12 cases have been documented (Evans et al., 2012), and these were invariably fatal.

Genetically, lyssavirus species can be separated into distinct phylogroups (Table 1.3 and Figure 1.4). Badrane et al. (2001) described two distinct phylogroups which separated rabies virus, DUVV, EBLV-1, EBLV-2 and ABLV into phylogroup 1 and MOKV and Lagos bat virus (LBV) into phylogroup 2 based on both genetic relationships and serological studies. A further putative phylogroup has been proposed (Kuzmin et al., 2005) which includes West Caucasian bat virus (WCBV), IKOV and LLEBV. Testing for VNAb against lyssavirus species is conducted using the same assays as for rabies virus, the RFFIT or FAVN, by simply substituting the test virus used in the assay. These VNTs are useful for measuring the antibody response if the lyssavirus responsible for infection is known, for example in

laboratory experiments or when the infecting virus has been identified by molecular techniques. However, cross-neutralising antibodies to lyssaviruses can obscure results in VNTs if the infecting lyssavirus is unknown and also during sero-epidemiological studies.

Antibodies raised against rabies virus cross-neutralise other phylogroup 1 lyssaviruses to varying degrees, but do not readily neutralise viruses from phylogroup 2 (Lafon et al., 1988, Brookes et al., 2005b, Hanlon et al., 2005, Wright et al., 2008, Badrane et al., 2001). The degree of neutralisation has been shown to correlate with the amino acid similarity of lyssaviruses (Badrane et al., 2001). Hanlon et al. (2005) demonstrated cross-neutralisation between two phylogroup 2 lyssavirus, LBV and MOKV, however no cross-neutralisation was observed to WCBV, a member of the putative third phylogroup. The cross-neutralising ability of rabies-specific VNAb across the phylogroup 1 viruses provides encouragement that rabies vaccination should protect against these viruses and therefore vaccination should be offered to those at risk of infection with phylogroup 1 lyssaviruses, such as laboratory workers and bat handlers. However, rabies vaccination would elicit little, if any, protection against phylogroup 2 viruses or those from the third phylogroup.

These cross-neutralisation patterns must be considered when interpreting serology from regions where lyssaviruses of the same phylogroup circulate, and indeed consideration must also be given to those as yet unidentified lyssaviruses that are undoubtedly circulating across the globe. Although VNABs may cross-neutralise a number of lyssaviruses, it has been documented that the highest VNAB titres are observed against the particular lyssavirus to which the initial immune response was elicited (Wright et al., 2008, Wright et al., 2010).

Pseudotype neutralisation tests have been described for LBV, MOKV, EBLV-1 and EBLV-2 (Wright et al., 2008, Wright et al., 2009). Serological assays that can be performed safely without the use of live virus are even more important for non-rabies lyssaviruses, as no specific vaccines are available for prophylaxis or post exposure treatment. An additional advantage of the pNT is that only the sequence for the glycoprotein is required, as opposed to a culture of live virus. Therefore, if a new lyssavirus is discovered the glycoprotein could be cloned directly from sequenced material, or even synthesised, negating the need to

culture large quantities of virus, which is time consuming and not always successful.

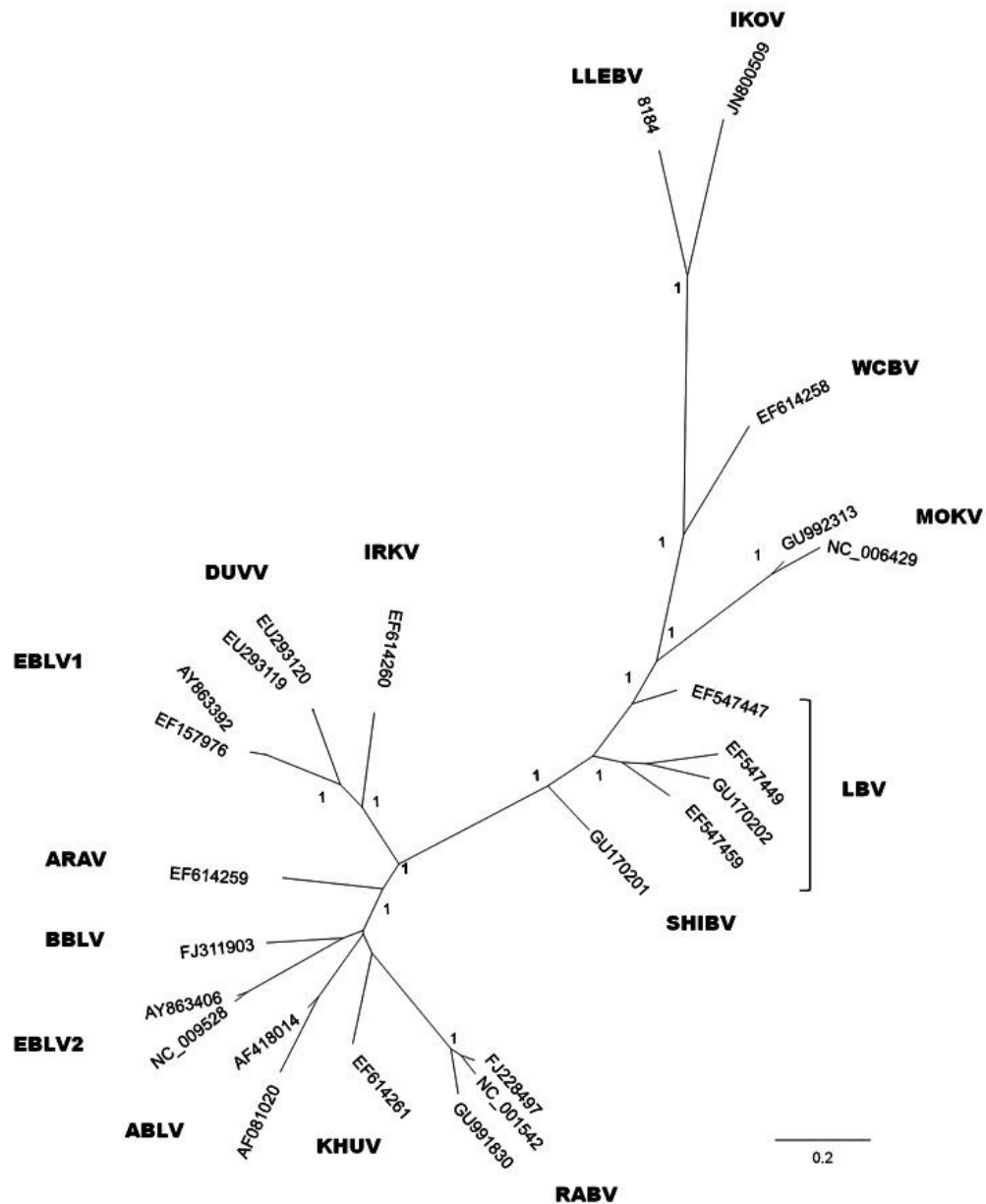


Figure 1.4 Phylogenetic tree of species of the Lyssavirus genus. Phylogenetic reconstruction based on the nucleoprotein gene of lyssaviruses, scale bar represents number of substitutions per site. Figure reproduced from Aréchiga Ceballos et al. (2013).

	Lyssavirus	Year identified	Country identified	Geographical distribution	Host species	Reference
Phylogroup 1	Duvenhage virus (DUVV)	1970	South Africa	Africa	Insectivorous bats	(Meredith et al., 1971)
	European bat lyssavirus type -1 (EBLV-1)	1977	Russia	Europe	Insectivorous bats	(Banyard et al., 2014)
	European bat lyssavirus type -2 (EBLV-2)	1985	Finland	Europe	Insectivorous bats	(Lumio et al., 1986)
	Australian bat lyssavirus (ABLV)	1996	Australia	Australia	Frugivorous bats	(Fraser et al., 1996)
	Aravan virus (ARAV)	1991	Kyrgyzstan	Eurasia	Insectivorous bats	(Kuz'min et al., 1992)

	Khujand virus (KHUV)	2001	Tajikistan	Eurasia	Insectivorous bats	(Kuzmin et al., 2003)
	Irkut virus (IRKV)	2002	Russia	Eurasia	Insectivorous bats	(Botvinkin et al., 2003)
	Bokeloh bat virus (BBLV)	2010	Germany	Europe	Insectivorous bats	(Freuling et al., 2011)
Phylogroup 2	Lagos bat virus (LBV)	1956	Nigeria	Africa	Frugivorous bats	(Boulger and Porterfield, 1958)
	Mokola virus (MOKV)	1968	Nigeria	Africa	Terrestrial mammals	(Shope et al., 1970)
	Shimoni bat virus (SHIBV)	2009	Kenya	Africa	Insectivorous bats	(Kuzmin et al., 2010)
Putative 3 rd	West Caucasian bat virus (WCBV)	2002	Russia	Eurasia	Insectivorous bats	(Botvinkin et al., 2003)

phylogroup	Ikoma lyssavirus (IKOV)	2009	Tanzania	Africa	African civet*	(Marston et al., 2012)
	Lleida bat lyssavirus (LLEBV)	2011	Spain	Europe	Insectivorous bats	(Aréchiga Ceballos et al., 2013)

Table 1.3 Geographical location and species associations of the non-rabies lyssaviruses. The date and place of virus identification, phylogroup, host species and geographical range of the 13 species (and the additional putative species, LLEBV) of non-rabies lyssaviruses. *IKOV has only been isolated from one animal to date.

1.4 Rift Valley fever virus

1.4.1 Rift Valley fever virus genome

Rift Valley fever virus was first described in Kenya in 1930, when a previously undescribed disease was affecting sheep farms: high mortality rates were observed in new born lambs and the majority of pregnant ewes aborted (Daubney et al., 1931). The cause of the disease was identified as RVFV, a member of the *Bunyaviridae* family and the *Phlebovirus* genus (King et al., 2012). The RVFV genome is around 12 kb in size and comprises three single stranded RNA segments; large (L), medium (M) and small (S). The L and M segments are negative sense whereas the S segment is of ambisense polarity. The L segment encodes for the viral RdRP, the M segment encodes for the glycoprotein precursor and the S segment encodes for the nucleoprotein and the non-structural NSs protein (Pepin et al., 2010) (Figure 1.5). The polyprotein precursor produced from the M segment is co-translationally cleaved into two glycoproteins, Gn and Gc, and two non-structural proteins (NSm1 and NSm2) (Gerrard and Nichol, 2007). The glycoproteins mediate virus entry into cells, however the specific receptors of RVFV remain to be identified (Harmon et al., 2012).

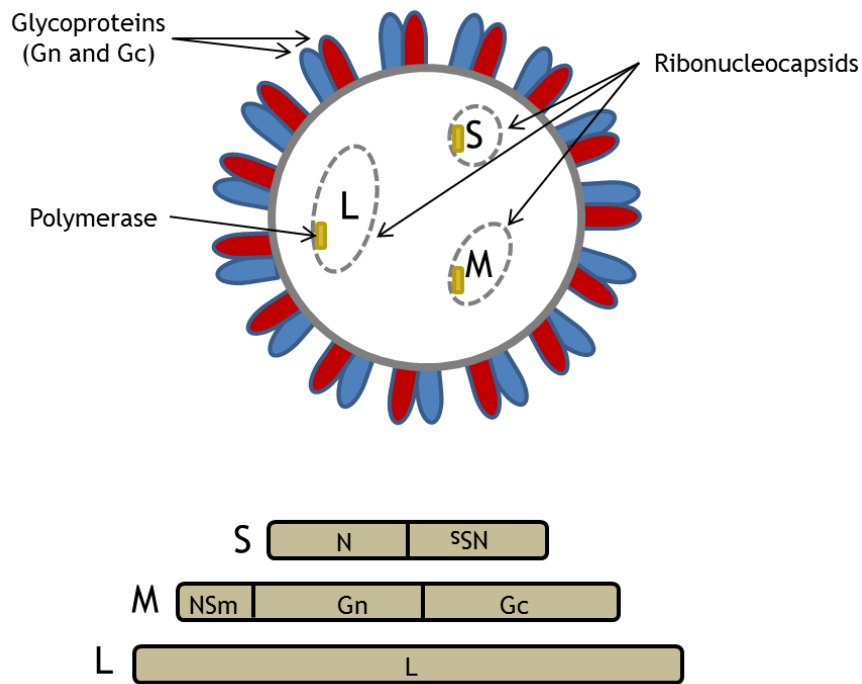


Figure 1.5 Schematic diagram of Rift Valley fever virus virion and the segmented genome. Schematic representation of a) the RVFV virion and b) the tripartite segmented genome. Figure adapted from Pepin et al. (2010).

1.4.2 Host species

The primary maintenance and transmission vectors of RVFV are mosquitoes of the *Aedes* genus, with mosquitoes of the *Culex* genus being implicated in transmission during outbreaks (Crabtree et al., 2012). However over 30 species of mosquito have been reported as being naturally infected with RVFV (Chevalier et al., 2010). Transmission cycles are complex, and differ between epidemics and through inter-epidemic periods (IEP). The leading hypothesis regarding viral maintenance between outbreaks involves the persistence of RVFV in diapause mosquito eggs which survive in shallow wetlands during IEP, although often quoted as ‘months’ there is very little natural or experimental data regarding the duration of such egg viability. Major outbreaks of RVFV tend to occur after heavy rainfall (Anyamba et al., 2009). It is hypothesised that following flooding of these wetlands, diapause eggs hatch releasing RVFV infected mosquitoes into the environment. Infected mosquitoes then transmit the virus to ruminants, who

act as amplifying hosts, and an explosive epidemic ensues. Mosquitoes transmit the virus to ruminants and humans, direct transmission from infected ruminants to other animals and humans is another major route of transmission during outbreaks and vertical transmission of RVFV has also been demonstrated in animals (Antonis et al., 2013). The role of wildlife hosts, both during outbreaks and IEP, is yet to be fully elucidated (Olive et al., 2012) (Figure 1.6). Rift Valley fever virus is predominantly considered a disease of domestic ruminants and humans, however clinical disease has also been documented in a variety of even-toed ungulate species such as camels (El Mamy et al., 2011), buffalo and waterbuck (Beechler et al., 2015). Rift Valley fever virus was confined to continental Africa until it was detected in Madagascar in 1979 (Carroll et al., 2011) and subsequently the virus has spread further, being detected in the Arabian Peninsula in 2000 (Balkhy and Memish, 2003).

Little is known about maintenance of the virus during IEP, and although RVFV has been shown to persist in mosquito eggs during such periods, there have been many studies suggesting that RVFV does in fact circulate at a low-levels between outbreaks in livestock, humans and wildlife (Sumaye et al., 2013, Gray et al., 2015, LaBeaud et al., 2008, Beechler et al., 2015, Wensman et al., 2015). Hence further sero-epidemiological studies are required to understand inter-epidemic transmission and maintenance of RVFV, to better advise on preparedness and management of RVFV.

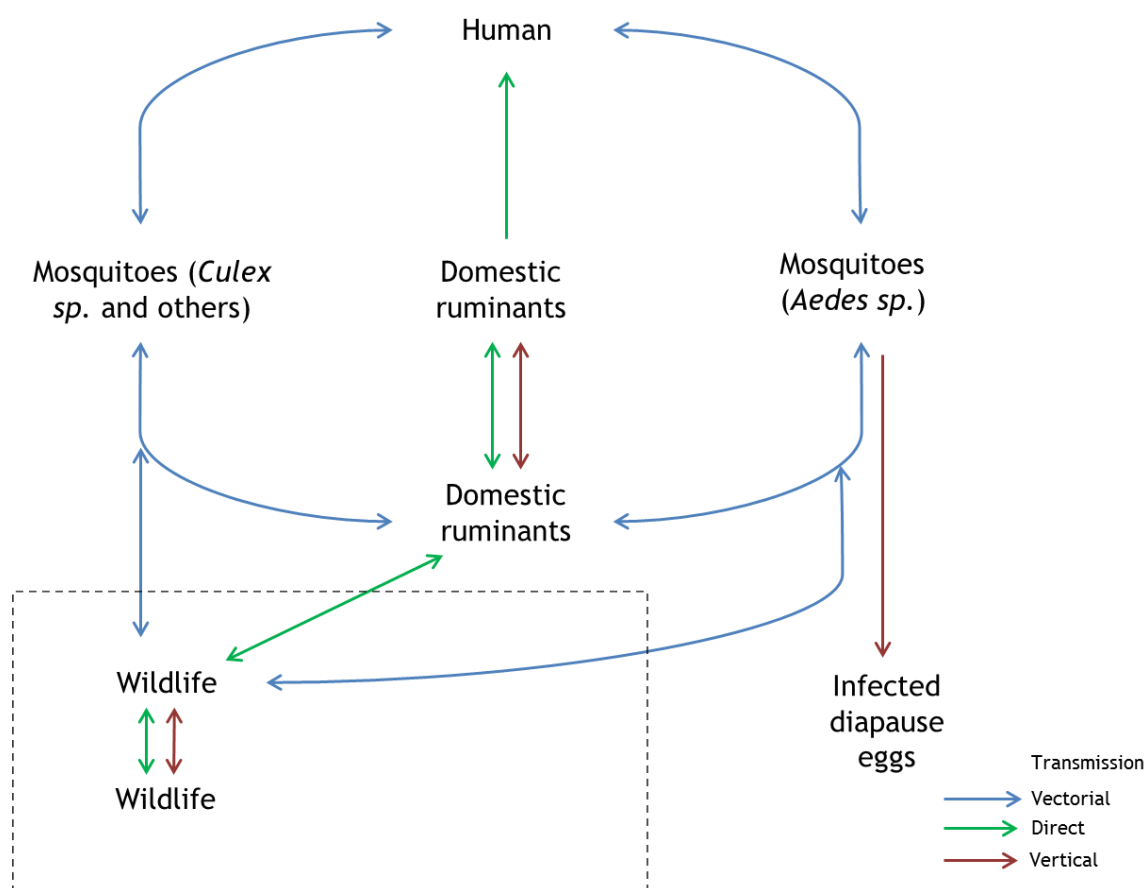


Figure 1.6 Proposed transmission cycle of Rift Valley fever virus. Transmission of RVFV between mosquito vectors, ruminant and human hosts. The role of wildlife in maintenance and transmission remains unclear.

1.4.3 Rift Valley fever virus infection

Sheep, and to a lesser extent cattle, are the principle hosts of RVFV in sub-Saharan Africa (Davies, 2010). Outbreaks of the disease are characterised by ‘abortion storms’ in domestic livestock and high mortality rates are observed in animals of less than one week of age. Clinical symptoms include pyrexia, mucopurulent nasal discharge, haemorrhagic diarrhoea, vomiting and jaundice. Onset of disease is sudden and death occurs within 12-24 h of developing symptoms. During outbreaks the mortality rate in animals of less than 10 days of age is 100% and 70% for lambs and calves respectively. Goats suffer the same clinical picture as sheep however present with less severe clinical symptoms and a lower mortality rate is observed (Davies and Martin, 2006).

In humans most RVFV infection causes a self-limiting febrile illness which lasts 3-4 days, however long term sequelae are observed in a minority of patients and can result in neurological manifestations and loss of vision (Ikegami and Makino, 2011). In rare cases RVFV infection involves haemorrhagic manifestations, patients present with fever, rigor, headaches, maculopapular rash, bleeding gums and bloody diarrhoea, death occurs 3-6 days after the onset of symptoms (Ikegami and Makino, 2011). No treatment is available for either the self-limiting or haemorrhagic forms of RVFV infection. Overall case fatality rates have historically been reported as around 1% (Spickler, 2010), however higher fatality rates of up to 47% have been recorded in more recent outbreaks (Mohamed et al., 2010). Human infection is increased in regions where consumption of milk and meat from sick animals is still practiced (Sindato et al., 2011).

During outbreaks the economic burden of RVFV is immense, the most affected communities tend to be the poorest, mainly farmers who rely on milk and meat production as both income and sustenance. These communities often live in remote areas with poor communication facilities, which means they are often unable to report RVFV outbreaks to the authorities (Fyumagwa et al., 2011). In addition to the immediate economic loss due to livestock deaths, bans on livestock trade during outbreaks also impact on livelihoods.

1.4.4 Vaccination

The development of effective and safe RVFV vaccines is required to protect humans and livestock during outbreaks. However, little progress in vaccine development has been made since the discovery of the virus and there is at present no licensed vaccine for use in humans (Ikegami and Makino, 2009). The first vaccine, produced in 1949 (Smithburn, 1949), is still in use in livestock today. This is a live-attenuated vaccine, which is inexpensive and provides long lasting immunity but has been reported to cause abortions and foetal abnormalities in pregnant animals (Botros et al., 2006). Inactivated whole-virus vaccines are also available and have a much higher safety rating than the live-attenuated vaccine, however these are expensive to produce and booster doses are required for optimal protection (Barnard, 1979).

1.4.5 Rift Valley fever diagnosis

Laboratory diagnosis of RVFV infection can be made by genome detection using real-time or gel based RT-PCR, however viraemia lasts only 3-4 days (Mansfield et al., 2015) hence the window for detection is limited. Therefore delays in hospitalisation or testing would result in the inability to detect circulating virus. Additionally, PCR requires specialist equipment and trained staff, which are often unavailable in countries where RVFV is endemic, hence the majority of RVFV diagnosis relies on detection of IgM antibodies. The immune response develops rapidly following RVFV infection, with IgM and IgG detectable from 4 and 8 days post infection respectively (Mansfield et al., 2015). Neutralising antibodies are raised primarily against the viral glycoproteins (Gn and Gc) (Besselaar and Blackburn, 1992) and are considered the best correlate of protection (Pepin et al., 2010). The majority of IgM and IgG antibodies are directed against the nucleoprotein, and to a lesser extent to the NSs (Pepin et al., 2010). The nucleoprotein is the major immunogen in RVFV infection and antibodies raised to the nucleoprotein are therefore utilised in the majority of serological assays. IgM antibodies are detectable for around 7 weeks (Niklasson et al., 1984) but there is little data regarding the duration of IgG or neutralising antibodies in natural infection.

The gold standard assay for assessing previous RVFV exposure, and for evaluating protective titres post vaccination, is the plaque reduction neutralisation test (PRNT) (OIE, 2012a). The PRNT detects neutralising antibodies, in sera of any species, and is considered the most specific RVFV antibody detection assay. However as the PRNT utilises live virus it requires BSL-3 containment facilities (HSE, 2013) which are often not available in RVFV endemic countries. Furthermore, the assay requires skilled technicians to perform and has an incubation period of up to 7 days, further hampering the use of this assay in the field. The most commonly utilised method for sero-epidemiology of RVFV is the ELISA (Mansfield et al., 2015), commercial RVFV ELISAs based on the viral nucleoprotein are available and detect IgM and/or IgG. The majority of these ELISAs are validated only for domestic ruminants and humans although a multi-species capture ELISA is also commercially available. These ELISAs allow for large scale serosurveillance outside of high containment facilities and are much more convenient for 'in field' testing. However many studies have demonstrated

poor agreement between PRNT and ELISA results in field samples (Evans et al., 2008, Gray et al., 2015, Kariithi et al., 2010). Furthermore, non-specific background has been observed using such assays which makes result interpretation difficult (Faburay et al., 2013). As these ELISAs detect antibodies raised to the RVFV nucleoprotein, further concern is raised with regard to antibody cross-reactivity, as antibodies raised against other members of the *Phlebovirus* genus have been shown to cross-react (Wu et al., 2014, Xu et al., 2007, Tesh et al., 1982, Szymczak et al., 2015). During the course of my PhD, I produced ELISAs based on the recombinant nucleoprotein of RVFV and two other phleboviruses; sandfly fever Sicilian virus (SFSV) and Toscana virus (TOSV) and demonstrated cross-reactivity of RVFV antibodies on SFSV and TOSV ELISAs (data not presented in this thesis). Although these viruses do not over-lap geographically with the current distribution of RVFV, there may be cross-reacting antibodies to as yet undocumented phleboviruses on the African continent.

1.4.6 Alternatives to conventional virus neutralisation tests

Kortekaas et al. (2011) demonstrated the production of replication deficient RVFV replicon particles (RRP) expressing GFP and performed a novel VNT using these RRP in place of live virus to test sera from experimentally infected sheep. These RRP were produced by transfecting a plasmid encoding the RVFV glycoproteins into a cell line which stably expressed the L and S segments of RVFV. The results of the VNT utilising RRP demonstrated 100% sensitivity and specificity when compared to results of the classical VNT, although only 11 samples were tested in their study. In 2014, Bukbuk et al. reported the production of RVFV pseudotypes produced using the VSV pseudotyping system, which were also successfully utilised as a substitute for live virus in a RVFV pNT.

1.5 Summary and statement of aims

Serological surveillance of viruses is fundamental for understanding infectious disease ecology. However, serological data can be difficult to interpret, especially when serological assays have imperfect sensitivity and specificities (Viana et al., 2016). The VNT is considered the gold standard for determining immunological response to either natural infection or vaccination; however these assays are seldom performed for sero-epidemiology of viruses that require handling at high containment. The majority of highly pathogenic zoonotic pathogens occur in parts of the world where such high containment facilities are seldom available, therefore routine serosurveillance of these pathogens are not performed, or are performed with inferior tests.

In this thesis I aimed to develop novel assays to measure VNAb to two important zoonotic viruses, rabies and RVFV. Specific, high-throughput, pan-species assays that can be performed outwith high containment facilities are required to produce robust serological data. Such data are essential to further understand the complex, multi-host ecology of these viruses.

In Chapter 3 the methods described previously by Wright et al. (2008) were used to optimise pseudotype neutralisation tests (pNT) for the detection of lyssavirus VNAb for use in our laboratory. In addition, pseudotypes were used to investigate the viral tropism of these lyssaviruses in a variety of adherent and suspension cell lines.

In Chapter 4 the rabies pNT optimised in Chapter 3 was used to investigate rabies VNAb titres in three studies. In the first study a serosurvey of domestic and wild animals in the Russian Far East was performed to investigate rabies infection patterns in an area in which there are plans to reintroduce the Amur leopard. The second study explores the use of rabies serosurveillance in wildlife (lions) from the Serengeti National Park (SNP) to provide information on rabies infection patterns in an area with an on-going rabies control and elimination programme. In the third study the rabies virus pNT was used to study the kinetics of rabies VNAb over a 14-month period in a cohort of vaccinated slum dogs from Kenya.

In Chapter 5 RVFV retroviral pseudotypes were produced and used to demonstrate their ability to detect VNAb in sera. The production of VSV pseudotypes expressing the glycoproteins of RVFV was also investigated. In addition, a novel RVFV neutralisation test was developed using a recombinant RVFV expressing luciferase.

In Chapter 6 the luciferase-expressing RVFV neutralisation test developed in Chapter 5 was used to detect RVF VNAb in three studies. In the first two studies, seroprevalence of RVFV in wildlife in the SNP was investigated, whilst in the third study the presence of RVF VNAb in a longitudinal cohort of domestic livestock from areas surrounding the SNP was examined.

Chapter 2. Materials and methods

2.1 Cell culture techniques

2.1.1 Cell lines

Human embryonic kidney 293T cells (293T, ATCC CRL-3216), baby hamster kidney cells (BHK-21, ATCC CCL-10), Crandell feline kidney cells (CRFK, ATCC CCL-94), and canine osteosarcoma D-17 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1mM sodium pyruvate. BSR-T7/5 cells, a BHK-21-derived cell line stably expressing T7 RNA polymerase, were maintained in Glasgow's Modified Eagle Medium (GMEM) supplemented with 10% FBS and 10% tryptose phosphate broth. Medium for 293T and BSRT-7/5 cells was supplemented with 400 µg/ml G418. BMOX40, MT-4, SUP-T1 and 3201 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1mM sodium pyruvate. All cell lines were grown at 37°C in a 5% CO₂ humid atmosphere. Cell lines were subcultured when approaching confluency. Adherent cell lines were subcultured by washing with phosphate buffered saline (PBS) and treating with 1ml of 0.05% trypsin-EDTA. Suspension cell lines were subcultured by centrifugation. Cells were then seeded at the required density. All media and supplements were obtained from Life Technologies Ltd., Paisley, UK. A summary of the cell lines used in this study is presented in Table 2.1.

Cell Line	Species	Adherent or suspension	Reference
293T	<i>Homo sapiens</i>	Adherent	(Graham et al., 1977)
3201	<i>Felis catus</i>	Suspension	(Hardy et al., 1980)
BHK-21	<i>Mesocricetus auratus</i>	Adherent	(Macpherson and Stoker, 1962)
BMOX40	<i>Canis familiaris</i>	Suspension	(Willett et al., 2006)
BSR-T7/5	<i>Mesocricetus auratus</i>	Adherent	(Buchholz et al., 1999)
CRFK	<i>Felis catus</i>	Adherent	(Crandell et al., 1973)
D-17	<i>Canis familiaris</i>	Adherent	(Riggs et al., 1974)
MT-4	<i>Homo sapiens</i>	Suspension	(Harada et al., 1985)
SUP-T1	<i>Homo sapiens</i>	Suspension	(Smith et al., 1984)
Vero	<i>Cercopithecus aethiops</i>	Adherent	(Yasumura and Kawakita, 1963)

Table 2.1 Cell lines used in this study.

2.1.2 Retroviral pseudotype production

293T cells were seeded 18 h prior to transfection at a density of 1×10^6 or 2×10^5 cells in either a 10 cm dish or per well of a 6 well plate respectively. Transfection was carried out using 1mg/ml polyethylenimine (PEI) (Polysciences) as the transfection reagent (Boussif et al., 1995). For transfection in a 10 cm dish 10 μ g of DNA and 60 μ l of PEI were added to 300 μ l of antibiotic and serum free DMEM or 2 μ g DNA, 10 μ l PEI and 100 μ l of antibiotic and serum free DMEM for transfection performed in 6 well plates. Transfection mixture was then vortexed and incubated at room temperature for 15 min to allow transfection-

complex formation. Transfection mixture was then added directly to the cells and incubated for 72 h at 37°C at 5% CO₂. Supernatants were harvested, centrifuged at 2000 rpm for 10 min, filtered at 0.45 µm and stored at -80°C prior to use. Pseudotypes were produced using ratios of 1:1.5:1 µg of gag-pol vector, reporter expression construct and glycoprotein respectively, unless otherwise stated. For two plasmid transfections a ratio of 1:1 µg was used. Reporter genes used in this study expressed the following proteins: green fluorescent protein (GFP), red fluorescent protein (RFP), β-galactosidase (*lacZ*) and luciferase (*luc*). Plasmids used for pseudotype production are presented in Table 2.2. Plasmids expressing viral glycoproteins are presented in Table 2.3. Plasmid pMDG is used to produce VSV pseudotypes, which are used as a positive control for both transfection and transduction. Empty expression plasmids VR1012 or pTM1 are used as negative controls for transduction, and referred to as 'ΔEnv', as there is no insert in place of the plasmid expressing the viral glycoprotein. VSV and ΔEnv pseudotypes are produced in parallel with each new batch of pseudotype, if there is any expression of reporter genes upon transduction of ΔEnv pseudotype then the test batch is not used as transduction cannot be confirmed as being glycoprotein mediated. All lyssavirus expression plasmids were kindly provided by Dr. Edward Wright (University of Westminster) and pTM1-GnGc was kindly provided by Prof. Richard Elliott (University of Glasgow).

For pseudotypes produced utilising recombinant vaccinia as the source of T7 polymerase, transfections were performed as previously described using CMVi, CNCG and pTM1-GnGc plasmids and incubated for 4 h. Equal volumes of 2×10^7 pfu/ml recombinant vaccinia virus (vTF7-5) and 0.5% trypsin were incubated at 37°C for 30 min, then added to the transfected cells and incubated for a further 24 h, pseudotypes were harvested as described previously.

Pseudotypes are denoted VIRUS(glycoprotein), for example MLV(rabies) indicates an MLV particle bearing the rabies virus glycoprotein.

Plasmid name	Function (reporter)	Viral origin	Reference
CMVi	gag-pol	MLV	(Towers et al., 2000)
HIT60	gag-pol	MLV	(Soneoka et al., 1995)
FP93	gag-pol	FIV	(Poeschla et al., 1998)
Fb-luc	gag-pol and reporter gene vector (firefly luciferase)	FIV	(Vaillancourt et al., 2002)
p8.2	gag-pol (+Vif)	HIV-1	(Naldini et al., 1996)
p8.91	gag-pol (-Vif)	HIV-1	”
HIV-eGFP	gag-pol and reporter gene vector (GFP)	HIV-1	(Van Parijs et al., 1999) (formerly referred to as pMIG)
pNL-Luc-E-R-	gag-pol and reporter gene vector (firefly luciferase)	HIV-1	(Connor et al., 1995)
CNCG	Reporter gene vector (GFP)	MLV	(Bock et al., 2000)
pMFG	Reporter gene vector (lacZ)	MLV	(Ohashi et al., 1992)
GinSin	Reporter gene vector (GFP)	FIV	(Poeschla et al., 1998)
LinSin	Reporter gene vector (Firefly luciferase)	FIV	”
CSRW	Reporter gene vector (DsRed)	HIV-1	(Naldini et al., 1996)
CSGW	Reporter gene vector (GFP)	HIV-1	”

Table 2.2 Plasmids used for retroviral pseudotype production.

Plasmid name	Expression plasmid	Glycoprotein	Reference
pMD.G	pMD	VSV	(Naldini et al., 1996)
LBV	pl.18	LBV	(Wright et al., 2009)
MOKV	pl.18	MOKV	”
DUVV	pl.18	DUVV	”
CVS-11	pl.18	Rabies virus	(Wright et al., 2008)
pl.18 RV9	pl.18	EBVL-1	”
pl.18 RV1787	pl.18	EBLV-2	”
pl.18 RV634	pl.18	ABLV	”
RVF-M	VR1012	RVFV (M segment)	This study
RVF-GnGc	VR1012	RVFV (GnGc polyprotein)	”
RVF-NSm1	VR1012	RVFV (NSM1 protein)	”
RVF-Gn	VR1012	RVFV (Gn)	”
RVF-Gc	VR1012	RVFV (Gc)	”
pTM1-GnGc	pTM1	RVFV (GnGc polyprotein)	(Carnec et al., 2014)
VR1012	VR1012	None	(Hartikka et al., 1996)
pTM1	pTM1	None	(Fuerst et al., 1986)

Table 2.3 Expression plasmids used for retroviral pseudotyping.

2.1.3 Rhabdoviral pseudotype production

Recombinant vesicular stomatitis viruses (VSV) in which the glycoprotein has been deleted (rVSV-ΔG) and replaced with GFP (rVSV-ΔG*GFP), RFP (rVSV-ΔG*DsRed) or luciferase (rVSV-ΔG**luc*) were used to produce VSV pseudotypes in this study. Recombinant VSV stocks were kindly provided by Dr. Michael Whitt (University of Tennessee). Reporter gene expressing recombinant VSV and their subsequent pseudotype production have been described (Whitt, 2010). Briefly, working stocks of reporter expressing rVSV-ΔG are generated by recovering infectious virus using a five plasmid reverse genetics protocol, after which the recovered virus is titrated onto cells expressing the glycoprotein of VSV *in trans*. Supernatants are then collected and purified using a plaque purification method.

Eighteen hours prior to transfection 293T cells were seeded at 2×10^5 cells per well in a 6 well plate. Transfection mixture (2 μg of glycoprotein expression vector, 10 μl PEI and 600 μl of antibiotic and serum free DMEM) was vortexed and incubated at room temperature for 15 min. Medium was removed from 293T cells and replaced with Opti-MEM (Life Technologies) whilst the transfection mixture was incubating. Opti-MEM was removed from the cells and transfection mixture was added to the cells and incubated for 4 h at 37°C. Supernatants were then removed and replaced with DMEM supplemented with 5% FBS and incubated for a further 24 h. Cells were then superinfected with $\sim 5 \times 10^5$ infectious units of rVSV-ΔG and incubated for 2 h at 37°C. Supernatants were removed and cells were washed 6-8 times with serum free DMEM, 1 ml of fresh DMEM was added to cells and further incubated for 24 h at 37°C. Supernatants were harvested, centrifuged at 2000 rpm for 10 min, filtered at 0.45 μm and stored at -80°C prior to use.

2.1.4 Pseudotype transduction

Target cell lines were seeded 18 h prior to transduction at a density of 1×10^5 cells or 1×10^4 cells per well in a 12 well or 96-well plate respectively. Pseudotypes were added to cell culture medium directly at the specified dilution and incubated at 37°C for 48 h, unless stated otherwise. Cells transduced with GFP or RFP expressing pseudotypes were examined using fluorescence microscopy or analysed by flow cytometry using the Accuri C6 flow cytometer (BD, Franklin Lakes, NJ, USA). The percentage of cells was calculated by

selecting an initial analysis gate on live cells, then a second analysis gate created on a histogram plot using non-transfected control cells, the positive cells were considered those which fell into the final gated region. For luciferase expressing pseudotypes, target cells were seeded 18 h prior to transduction at 1×10^4 cells per well in 50 μ l volumes in a white 96-well CulturPlate (Perkin-Elmer, Waltham, MA, USA), 50 μ l of pseudotype was added and incubated at 37°C for 48 h after which 100 μ l of Steadylite Plus luciferase substrate (Perkin Elmer) was added and luciferase expression analysed on a Microbeta 1450 Jet luminometer (Perkin Elmer). Cells transduced with pseudotypes expressing *lacZ* were visualised by X-gal staining. Cells were washed in PBS before being fixed with 0.5% glutaraldehyde for 30 min, fixative solution was removed and cells re-washed before the addition of staining solution (PBS containing 0.02% X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 3 mM ferro-cyanide and 1.3 mM $MgCl_2$). Plates were stored at 4°C overnight before *lacZ*-expressing cells were counted manually.

2.1.5 Quantification of pseudotype titre

To determine the infectious titre of GFP pseudotypes the 50% tissue culture infective dose ($TCID_{50}$) was calculated for each new batch of pseudotype generated and was specific to each batch of recovered cells. To calculate $TCID_{50}$, cells were seeded at a density of at 1×10^4 cells per well in a 96-well plate 18 h prior to addition of 100 μ l volumes of doubling dilutions of pseudotype in quadruplicate. Cells were incubated at 37°C for 48 h and examined by fluorescence microscopy and wells were marked as positive if one or more fluorescent cell was observed. The $TCID_{50}$ was calculated using the Spearman-Kärber formula (Hierholzer and Killington, 1996).

2.2 Molecular cloning techniques

2.2.1 Polymerase chain reaction

Five sections of the RVFV M segment were amplified from a plasmid expressing the M segment of the MP-12 strain of RVFV. The plasmid pTVT7R-M(MP12) (Billecocq et al., 2008) was kindly provided by Dr. Michelle Bouloy (Institut Pasteur). Amplified sections corresponded to: the entire ORF of the M segment

of RVFV, GnGc, NSm1, Gn and Gc. RVFV expression plasmids cloned in this study are summarised in Table 2.4. Primers were designed, and numbering of nucleotides was based on the published RVFV MP-12 sequence (GenBank accession number DQ380208). Primers were manufactured by Eurofins MWG, Germany. Polymerase chain reaction (PCR) was performed using High Fidelity PCR Master (Roche, UK) using the following thermocycling conditions: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 65°C for 60 s and extension at 72°C for 210 s, with a final extension at 72°C for 7 min. Thermocycling was conducted using a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, UK).

Plasmid name	Expressed protein	Nucleotide position	Primer sequences (5'-3')	
RVF-M	M segment	21-3614	F	ACTGGTCGACATGTATGTTTTA TTAACAATTCTAACCTCG
			R	ACAGCGGCCGCCTATGAGGCCT TCTTAGTGGC
RVF-GnGc	GnGc polyprotein	411-3614	F	ACTGGTCGACATGGCAGGGATT GCAATGACAG
			R	ACAGCGGCCGCCTATGAGGCCT TCTTAGTGGC
RVF-NSm1	NSm and Gn fusion protein	21-2090	F	ACTGGTCGACATGTATGTTTTA TTAACAATTCTAACCTCG
			R	ACAGCGGCCGCCTATGCTGATG CATATGAGAC
RVF-Gn	Gn glycoprotein	411-2090	F	ACTGGTCGACATGGCAGGGATT GCAATGACAG
			R	ACAGCGGCCGCCTATGCTGATG CATATGAGAC
RVF-Gc	Gc glycoprotein	2091-3614	F	ACTGGTCGACATGTGTTTCAGAA CTGATTCAGGC
			R	ACAGCGGCCGCCTATGAGGCCT TCTTAGTGGC

Table 2.4 Rift Valley fever virus expression plasmids generated in this study. Expression plasmids were generated by cloning sections of the RVFV M segment into mammalian expression vector VR1012. Nucleotide positions are designated with reference to the RVFV MP-12 sequence (GenBank accession number DQ380208). F and R denote forward and reverse primer sequences used to amplify RVFV genes.

2.2.2 Restriction enzyme digestion and ligation

The PCR amplification introduced Sall and NotI restriction sites at the 5' and 3' ends of the genes to facilitate cloning into the mammalian expression vector VR1012. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide, DNA bands were observed on a UV transilluminator (Ultra-Violet Products Ltd., Upland, CA, USA) and excised using a sterile scalpel. Gel extraction was performed using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified PCR products and VR1012 were digested in 20 µl reactions using 1 µl Sall (15 U/µl), 1 µl NotI (10 U/µl), 2 µl 10x Buffer H, 2 µl 0.10% BSA (Invitrogen), 5 µl DNA and 9 µl sterile water and incubated at 37°C for 2 h. Digested insert DNA was purified using QIAquick PCR purification kit (Qiagen) and digested VR1012 DNA was purified from agarose gel as described above. Vector and insert DNA were ligated overnight at 16°C using T4 DNA ligase (Life Technologies).

2.2.3 Transformation

DNA was transformed into DH5α MAX efficiency competent cells (Invitrogen). Competent cells (50 µl) were thawed on ice and 5 µl of ligation product was added, cells were incubated on ice for 30 min. Competent cells were then heat shocked in a water bath at 42°C for 40 s. Cells were returned to ice for a further 2 min, after which 400 µl of S.O.C media was added and incubated at 37°C for 1 h whilst being shaken on an orbital shaker. Cultures were spread on to LB agar plates containing 50 µg/ml kanamycin (Sigma-Aldrich, UK) and incubated at 37°C overnight. Individual bacterial colonies were added to 3 ml LB nutrient broth with kanamycin, broths were incubated overnight at 37°C on an orbital shaker.

Plasmid DNA was extracted from cultures using QIAprep Spin Miniprep kit (Qiagen) and digested as above. Clones containing inserts were identified by agarose gel electrophoresis, 1ml of each positive culture was inoculated into 500ml of LB nutrient broth with kanamycin and grown overnight at 37°C on an orbital shaker. Plasmid DNA was isolated from large scale cultures using a PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen). Pelleted DNA was resuspended in TE buffer and stored at 4°C. All plasmids were verified by Sanger sequencing prior to use (GATC Biotech, Konstanz, Germany).

2.2.4 Western blotting

Western blots were performed to detect expression of proteins in both cell lysates and concentrated pseudotypes. Transfected 293T cells were washed in PBS, resuspended in 75 µl NP-40 lysis buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris, pH 8.0) and incubated on ice for 30 min. 25 µl of SDS-PAGE loading buffer (40% Glycerol, 8% SDS, 5% β-mercaptoethanol, 0.04% bromophenol blue, 240 mM Tris/HCl, pH 6.8) was added to each sample. Samples were then sonicated for 30 s, incubated at 90°C for 10 min and centrifuged at 14000 rpm for 10 min. Pseudotypes were concentrated by centrifugation through a sucrose cushion. 1 ml of pseudotype was added to 400 µl of cold 20% sucrose and centrifuged for 1 h at 4°C. Supernatants were removed and pellets washed in 1 ml PBS by centrifugation, after which 40 µl of SDS-PAGE loading buffer was added to each sample. Samples were loaded in to NuPAGE 4-12% Bis-Tris precast polyacrylamide gels (Life Technologies) for electrophoresis at 2 h at 100 V in MES SDS running buffer (Life Technologies). Proteins were transferred to PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 1x casein solution (blocking buffer) (Vector Laboratories Ltd., Peterborough, UK) for 2 h. Primary antibodies were diluted in blocking buffer to 1:1000 and incubated at room temperature for 1 h. Membranes were washed in PBS-Tween three times prior to incubation with the secondary antibody. Biotinylated horse anti-mouse IgG antibody (Vector Laboratories Ltd.) was diluted in blocking buffer to 1:1000 and incubated at room temperature for 1 h, then washed in PBS-Tween three times prior to development. The Vectastain ABC-Amp Immunodetection kit (Vector Laboratories Ltd.) was utilised for visualisation of proteins, a preformed streptavidin/biotinylated alkaline phosphatase complex was incubated with the

membrane for 20 min, washed in PBS-Tween three times prior to addition of the chromogenic alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium), which was incubated in the dark until sufficient protein staining was observed.

The primary antibodies used to detect rabies virus and RVFV glycoproteins were the mouse anti-rabies virus glycoprotein clone IC5 (Bio-Rad Laboratories) and the mouse monoclonal anti-RVFV Gn glycoprotein clone 4D4 (Battles and Dalrymple, 1988) kindly provided by Dr. Felix Kreher (University of Glasgow). A cell lysate of A549 cells infected for 72 h with the MP-12 strain of RVFV was used as positive control material for RVFV western blots and was kindly provided by Dr. Ben Brennan (University of Glasgow).

2.3 Antibody detection assays

2.3.1 Lyssavirus pseudotype neutralisation test

In a 96-well plate 85 μ l of 100 TCID₅₀ MLV(lyssavirus) pseudotype was added to 85 μ l of doubling dilutions of sera (final concentrations of 1:10 to 1:640 were used as standard, although sera were tested at higher dilutions where required). 25 μ l of 100 TCID₅₀ MLV(VSV) pseudotype was added to 25 μ l of test serum diluted at the three lowest concentrations (final concentrations 1:10, 1:20, 1:40). Serum/pseudotype mixtures were incubated at 37°C for 1 h to allow neutralisation to occur, 50 μ l of serum/pseudotype mix were added to CRFK cells in triplicate. Cells had been seeded at a density of at 1×10^4 cells per well in a 50 μ l volume in 96-well plates 18 h previously. Cells were incubated at 37°C for 48 h and examined by fluorescence microscopy, wells were marked as positive if one or more fluorescent cell was observed. The VNAb titre was recorded as the reciprocal of the highest dilution at which at least two of the three wells were negative for fluorescent cells. This interpretation is based upon the RFFIT protocol provided by OIE, which states that the end-point titre is defined as the dilution factor of the highest serum dilution at which 50% of wells are neutralised (OIE, 2012a). However, in this assay the serum was tested in triplicate, which both reduced the volume of serum required and also allowed four samples to be tested per plate, therefore the titre was defined as the

dilution factor of the highest serum dilution at which >50% of wells were neutralised.

With each assay performed three control sera were tested in parallel. Two canine sera which had previously been tested by the FAVN (Wright et al., 2009) and measured >640 and <10 on the rabies virus pNT were used as positive and negative controls respectively. A further sample from a vaccinated human, which had been diluted to measure 40 on the rabies virus pNT, was also used.

An aliquot of the OIE standard reference dog serum, diluted to 0.5 IU/ml was kindly provided by Dr. Daniel Horton (Animal Health and Veterinary Laboratories Agency).

Each serum sample was individually tested for non-specific neutralisation using MLV(VSV) pseudotypes. Dilutions of sera up to 1:40 are incubated with MLV(VSV) pseudotypes, if rabies VNAb titre was >20 and the VSV control was also recorded as >20, the sample was repeat tested with MLV(VSV) pseudotypes with further serum dilutions of up to 1:640, in order to detect non-specific neutralisation. If rabies VNAb titre was four-fold higher than the VSV titre then the rabies VNAb titre was reported, if not the sample was recorded as inconclusive, as non-specific neutralisation cannot be excluded as the cause of the result.

2.3.2 Recombinant RVFV expressing *Renilla* luciferase virus neutralisation test

The recombinant virus rMP12ΔNSs:hRen was kindly provided by Dr. Ben Brennan (University of Glasgow). This virus was generated using a recombinant rMP12 S segment in which the NSs coding sequence was replaced with that of *Renilla* luciferase (Brennan et al., 2011). This altered S segment was subsequently used in the generation of a recombinant RVFV rMP12ΔNSs:hRen, by rescue of infectious rMP-12 as described by Billecocq et al. (2008). The recombinant virus rBUNΔNSs:hRen (Dietrich et al., 2016) was kindly provided by Dr. Xiaohong Shi (University of Glasgow).

In a 96-well plate 55 µl of rMP12ΔNSs:hREN containing 8×10^4 PFU in PBS/2% FBS was added to 55 µl of a 1:50 dilution of test sera (final concentration of 1:100). Serum/virus mixtures were incubated at 37°C for 1 h to allow neutralisation to occur. Media was removed from BHK-21 cells, seeded 18 h previously at a cell density of 1×10^4 cells per well, and 50 µl of serum/virus were added to cells in

duplicate. Virus was adsorbed onto cells for 1 h at 37°C before being removed and replaced with 100 µl culture media. Cells were then incubated for a further 5 h, after which medium was removed and cells were lysed in 100 µl passive lysis buffer (Promega, Madison, WI, USA) and left at room temperature for 10 min. 45µl of cells were transferred to a white flat bottomed 96-well plate containing 45µl of *Renilla*-Glo reagent (Promega), sealed and incubated at room temp for 10 min. Plates were analysed on a luminometer using the Glomax software and the *Renilla*-Glo Promega protocol, with integration time at 10 s per well.

With each assay performed, positive and negative control sera were tested in parallel, in addition to a 'cell only' control and a 'virus only' control to allow for calculation of percent neutralisation. All assays were performed at containment level three under the supervision of either Dr. Stephen Welch or Prof. Brian Willett.

2.3.3 Plaque reduction neutralisation test

The plaque reduction neutralisation test (PRNT) was performed using BHK-21 cells, seeded 18 h prior to infection in 12 well plates at a cell density of 7×10^5 cells per well. The monoclonal anti-Gn RVFV neutralising antibody (4D4) was serially diluted in PBS/2% FBS from 1:2 to 1:2048 and 200 µl of diluted antibody was incubated with 200 µl rMP-12 (in cell culture medium containing 480 PFU/ml) for 1 h at 37°C, to allow neutralisation to occur. After which 150 µl of antibody/virus mix was added to each well and incubated for 1 h at 37°C, the inoculum was then removed and replaced with overlay medium comprising GMEM supplemented with 2% FBS and 0.6% Avicel (FMC Corporation, Philadelphia, PA, USA). Cells were incubated for 72 h at 37°C, then fixed in 8% formaldehyde for at least 4 hrs before the plaques were visualized by staining with crystal violet solution. The PRNT was performed by Dr. Felix Kreher (University of Glasgow).

2.3.4 Rift Valley fever recN IgG ELISA

African buffalo samples were tested using the RVF recN IgG indirect ELISA (BDSL, Ayrshire, Scotland) which were performed according to the manufacturer's instructions.

2.4 Serum samples

All serum samples were heat inactivated at 56°C for 30 min prior to testing.

2.4.1 Tanzanian canine sera (tested in 3.2.2)

Serum samples from domestic dogs living in the Serengeti district of Tanzania were collected during the annual vaccination campaign undertaken by the Viral Transmission Dynamics Project. Samples were collected immediately prior to vaccination against rabies virus (Nobivac® Rabies, Intervet, Netherlands). Additional samples were collected 20-21 days post vaccination. A selection (n=104) of sera from these dogs were used in this study and had previously been tested for rabies VNAb using the FAVN at the Animal Health and Veterinary Laboratories Agency, Weybridge and also using the HIV(luc) pNT at University College London (Wright et al., 2009).

2.4.2 Russian wildlife and domestic animal sera (tested in 4.2.1)

Sera for testing were provided by Martin Gilbert (Wildlife Conservation Society, New York) and Dr. John Lewis (International Zoo Veterinary Group) (n=203). Samples had been opportunistically collected between 1989 and 2010 and prior to testing had been stored at -70°C.

2.4.3 Tanzanian lion sera (tested in 4.2.2 and 6.2.1)

Lion sera (n=107) were collected opportunistically from animals within the Serengeti ecosystem between 1997 and 2011 during disease monitoring operations, veterinary interventions (e.g. snare removals) and research activities (e.g. fitting radiocollars). Samples were collected by the veterinary units of Tanzania National Parks (TANAPA) and the Tanzania Wildlife Research Institute (TAWIRI). Samples were stored in an archived serum bank maintained at the University of Glasgow.

2.4.4 Kenyan slum dog sera (tested in 4.2.3)

Serum samples were available from an archive of samples originally collected from dogs from the Kibera slum in Nairobi, Kenya in 2007. The original samples formed part of a large study to assess the use of dogs in Kenya as sentinels of infectious diseases (Halliday, 2010). As an incentive to dog owners to participate

in the study, dogs were vaccinated against rabies virus at enrolment (Rabisin, Merial Ltd., France). A selection of dogs (n=67) from the original study were included for use in this study.

2.4.5 UK canine sera (tested in 4.2.3.1)

Thirty anonymised canine serum samples, which had been sent to the University of Glasgow Veterinary Diagnostic Services for various serological tests, were kindly provided by Michael McDonald. These samples were presumed to be from dogs born and bred in the UK. No history was available in relation to vaccination status or travel.

2.4.6 Tanzanian lion sera (tested in 5.2.3)

Nineteen lion samples, collected as part of ongoing lion surveillance programmes for canine distemper virus in the Serengeti National Park (SNP) in 1998, were kindly provided by Prof. Craig Packer (University of Minnesota). These samples had previously been tested for RVF VNAb using the PRNT by Prof. Clarence Peters (University of Texas Medical Branch).

2.4.7 African buffalo and gazelle sera (tested in 5.2.3 and 6.2.2)

Serum samples collected from buffalo (n=99) and gazelle (n=74) were available from an archived bank of sera collected from the SNP and Ngorongoro conservation area between 1993 and 2012. Sera had originally been collected as part of ongoing disease surveillance programmes for foot-and-mouth disease and peste des petits ruminants. A selection of these samples (n=34) had previously been tested for RVFV antibodies using the ID SCREEN RVFV IgM capture ELISA (ID Vet, Grabels, France) at The Pirbright Institute.

2.4.8 Tanzanian longitudinal livestock sera (tested in 6.2.3)

Sera were collected as part of a longitudinal study of livestock from the Serengeti district and Loliondo areas of Tanzania in 2009-2010. The original project was coordinated, and sera were kindly provided, by Dr. Harriet Auty (Scotland's Rural College). Animals enrolled in the study were ear tagged at recruitment so they could be individually identified throughout the study period. Aging of livestock was performed by consultation with the owners and then checked by dental aging. Animals had not been previously vaccinated against

RVFV. All field sampling was conducted by a Tanzanian veterinarian in collaboration with Sokoine University of Agriculture. Samples were stored at -20°C at the Nelson Mandela African Institute of Science and Technology, until exported to the University of Glasgow.

2.5 *In silico* techniques

2.5.1 Phylogenetic analysis

Nucleotide sequences which correlated to the glycoprotein sequence of the plasmid used to produce lyssaviruses pseudotypes in this study were gathered from GenBank. Accession numbers: CVS-11 (EU352767), LBV (EF547428), MOKV (GQ500108), DUVV (EU623444), EBLV-1 (EU352768), EBLV-2 (EU352769), ABLV (AF426311). VSV-G was used as an outgroup (V01214). Sequences were aligned using ClustalW (Larkin et al., 2007). A phylogenetic tree was produced in MEGA6 (Tamura et al., 2013), using the Neighbour-Joining method (Saitou and Nei, 1987) with 1000 replicates to allow calculation of bootstrap values. Evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004). The tree is to scale and branch lengths indicate the number of base substitutions per site.

Pairwise amino acid distances were calculated using the maximum composite likelihood model in MEGA6 (Tamura et al., 2013) using the ectodomain sequences of the lyssaviruses.

2.5.2 Graphs and statistics

Graphs were constructed using GraphPad Prism 6 (GraphPad Software, La Jolla California, USA,). Where shown, error bars represent the standard deviation unless otherwise stated. Sensitivity was calculated using the formula $TP/(TP+FN) \times 100$ and specificity using $TN/(TN+FP) \times 100$, where TP are true positives, TN are true negatives, FP are false positives and FN are false negatives. 95% confidence intervals were calculated using the Clopper-Pearson method.

2.5.3 Statistical models

Statistical modelling was completed using R version 3.1.1 (R, 2014). Correlates of continuous response variables were investigated using general linear models. Correlates of binary response variables were investigated using generalised linear mixed-effects models fitted using the *glmm* package and the function `glmm()` and generalised linear models fitted using the base R function `glm()`. Pairs of nested models were compared using likelihood ratio tests (LRTs) and p-values reported result from these tests. Model quality was also assessed through calculation of AIC scores (Akaike, 1974). For generalised linear mixed-effects models LRTs were conducted using the `anova()` function. As `anova()` cannot be used for LRTs of models fitted using `glm()`, LRTs were instead performed manually by calculation of log likelihood scores and the `pchisq()` function.

2.5.4 Geographical information system maps

Maps in Figures 6.2 and 6.4 were produced using GPS map coordinates imported into QGIS (QGIS, 2016) combined with base map data. The QGIS project file was then imported into Adobe Illustrator to produce the final figures. Both figures were kindly produced by Mr. Mike Shand (University of Glasgow).

Chapter 3. Development of lyssavirus pseudotype neutralisation tests

3.1 Introduction

Rabies virus belongs to the *Lyssavirus* genus, a member of the *Rhabdoviridae* family. Lyssaviruses are neurotropic viruses which can cause diseases in all mammals including humans. Once symptoms occur the disease has an almost 100% case fatality rate. Although rabies has been eliminated in most developed countries, it is still the cause of around 59,000 human deaths annually (Hampson et al., 2015). The majority of these deaths are caused by canine rabies in Asia and Africa, with bat rabies being the principal source of infection in the Americas. In humans, rabies has the highest fatality rate of all known viruses, however the disease in humans is entirely preventable through effective vaccination. This includes pre-exposure vaccination of people at risk and post-exposure prophylaxis administered immediately after the bite of a suspected rabid animal (PHE, 2013).

The primary correlation of protection against rabies virus is the presence of rabies virus neutralising antibodies (VNAb) (Johnson et al., 2010), which can occur through natural exposure or post vaccination. Virus neutralisation tests (VNT) are the preferential method of testing for rabies VNAb, with the fluorescent antibody virus neutralisation test (FAVN) and the rapid fluorescent focus inhibition test (RFFIT) being the two methods prescribed by the World Organisation for Animal Health (OIE) for international trade (OIE, 2012b). Both the FAVN and RFFIT require the use of live rabies virus, which is a Category 4 pathogen under the Specified Animal Pathogens Order 2008 (SAPO) and necessitates high containment facilities for virus handling. This limits the use of the current VNTs in endemic areas within developing countries, both because of a lack of high containment laboratories and the high cost of these assays. Other

available assays, such as ELISA-based tests, lack the sensitivity of the FAVN (Wasniewski et al., 2014).

Wright et al. (2009) have previously described a rabies VNT in which live virus is replaced with viral pseudotypes. Neutralisation assays utilising pseudotypes as a substitute for live virus have also been described for other high containment viruses including chikungunya, Japanese encephalitis virus and highly pathogenic strains of influenza A (Hu et al., 2014, Lee et al., 2014, Temperton et al., 2007). Pseudotyping involves co-expressing the glycoprotein of the virus of interest, with vectors encoding retroviral gag and pol genes. The resulting pseudotypes are infectious but replication defective, therefore can be handling at biosafety level 2 (BSL-2). A reporter gene, such as that encoding green fluorescent protein (GFP) can also be expressed in the pseudotype, allowing easy detection and the potential for absolute quantification.

The aim of this study was to produce viral pseudotypes bearing a GFP reporter gene, from seven species of lyssavirus and to assess their ability to replace live virus in a pseudotype-adapted neutralisation test. These pseudotype neutralisation tests (pNT) could provide a robust, cost-effective and flexible method of measuring lyssavirus VNAb.

3.2 Results

3.2.1 Production of GFP pseudotypes expressing the rabies virus glycoprotein

Rabies virus glycoprotein was incorporated into MLV particles to produce high titre rabies glycoprotein bearing MLV pseudotypes (MLV(rabies)) following protocols established previously in our laboratory (Stewart et al., 2012). Briefly, pseudotypes were produced by three plasmid co-transfection; a CMV promoter-driven MLV gag-pol packaging vector; CMVi, an MLV vector encoding a GFP reporter gene; CNCG, and a plasmid expressing the glycoprotein of the CVS-11 strain of rabies virus. Pseudotypes were then transduced into target cells where GFP expression was observed (Figure 3.1).

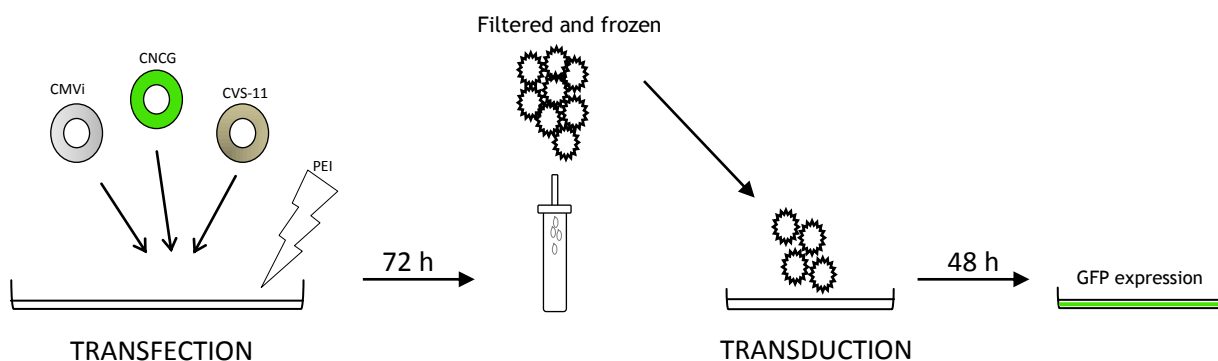


Figure 3.1 Schematic representation of MLV(rabies) pseudotype production. A three plasmid system was adopted to generate pseudotypes expressing the rabies virus glycoprotein. An MLV gag-pol packaging construct, a construct expressing GFP and a plasmid bearing the rabies virus glycoprotein were transfected, using PEI, into 293T cells. After 72 h the supernatant was harvested, filtered and frozen. Pseudotypes were then transduced into a target cell line where GFP expression is used to measure infectivity.

Initial work on pseudotypes in our laboratory has highlighted the importance of concurrently producing positive and negative controls when generating the rabies virus pseudotypes. These are required to control the process of pseudotype production, including transfection cell line quality, transfection efficiency and to detect any non-specific fluorescence seen in transduced cells. Therefore with each batch of MLV(rabies) pseudotype produced, additional positive and negative control pseudotypes are produced in parallel. Vesicular stomatitis virus glycoprotein (VSV) was used as a positive control as VSV pseudotypes are produced to high titres and are able to mediate cell entry into all cell lines currently studied (Coil and Miller, 2004). A negative control was produced by transfecting a plasmid containing no insert in place of the plasmid expressing the viral glycoprotein; these pseudotypes are referred to as “ Δ Env”. During this study we found that certain batches of 293T cell lines used for transfection produced Δ Env pseudotypes that expressed GFP in the target cell line (data not shown). These ‘bald particles’ have been documented previously (Voelkel et al., 2012) and therefore rabies virus pseudotypes were only used

when the concurrently produced Δ Env negative control pseudotype produced no GFP expression upon transduction, ensuring the GFP expression observed in the transduced cell line is mediated entirely by the glycoprotein on the pseudotype membrane.

Western blots were performed to confirm the presence of rabies virus glycoprotein in the pseudotypes. Protein expression was analysed in both cells transfected with CMVi, CNCG and rabies virus glycoprotein expression vector and the resulting pseudotypes generated. A protein of approximately 58 kDa correlating to rabies virus glycoprotein was expected, however no such protein band was visualised by Western blot analysis. Although a band of approximately the same size was present in the MLV(rabies) pseudotypes (lane 2), a non-specific band was also present in the Δ Env pseudotype (lane 4) (Figure 3.2). Additionally, we did not have any live virus infected cell lysate to act as a positive control for the Western blot analysis. The anti-rabies virus glycoprotein primary antibody used was not validated for use in Western blots and therefore we could not assume the protein was not being expressed, however antibodies to rabies virus glycoprotein which had been used successfully in Western blots by other studies (Koraka et al., 2014, Wright et al., 2008) were not available. Specificity of the MLV(rabies) pseudotypes at this point in the study were alternatively confirmed by specific neutralisation of serum known to be positive for rabies VNAb (data not shown).

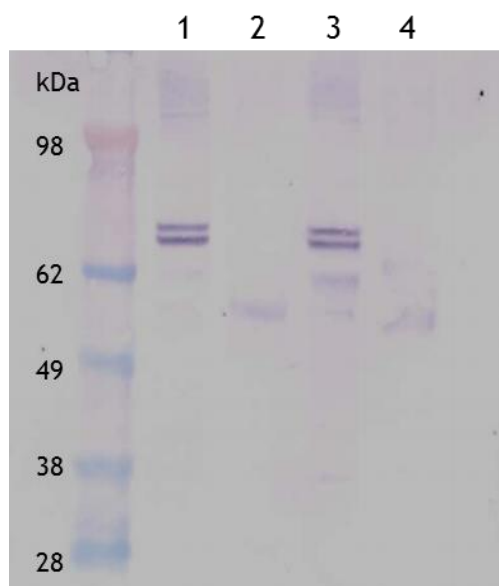


Figure 3.2 Expression of rabies virus glycoprotein in pseudotypes and producer 293T cells. Western blot analysis of rabies virus glycoprotein in 293T cells and concentrated cell lysates were performed to confirm rabies virus glycoprotein expression. Transfected 293T cells used to produce rabies virus pseudotypes (lane 1) and the resulting concentrated MLV(rabies) pseudotype particles (lane 2) were analysed in addition to negative controls (Δ Env) in which cells were transfected with an empty expression vector in place of the rabies virus glycoprotein expression vector. Transfected cell lysate (lane 3) and supernatant (lane 4) of Δ Env transfections. Membranes were probed with mouse anti-rabies virus glycoprotein antibody.

The infectivity of these pseudotypes was investigated by transduction into CRFK cells, a cell line shown to be susceptible to rabies virus pseudotypes in previous experiments performed in our laboratory. Fluorescence microscopy was performed, cells transduced with both MLV(rabies) and MLV(VSV) pseudotypes expressed GFP, whereas no GFP expression was observed in cells transduced with Δ Env pseudotype (Figure 3.3). As Δ Env pseudotypes were produced concurrently with the MLV(rabies) pseudotypes, we can assume that the lack of GFP expression was due to a lack of glycoprotein mediated cell entry. GFP expression decreased in a titratable fashion as the pseudotype concentration was decreased (Figure 3.4), demonstrating that these high titre pseudotypes

could be diluted significantly and still contain viable pseudotype. This is an important consideration, as the virus concentration employed in the FAVN is a highly diluted dose of 30-300 TCID₅₀ per well (OIE, 2012b). If a similar concentration was used in our neutralisation assay, one round of MLV(rabies) pseudotype production would supply enough pseudotype for hundreds of neutralisation tests.

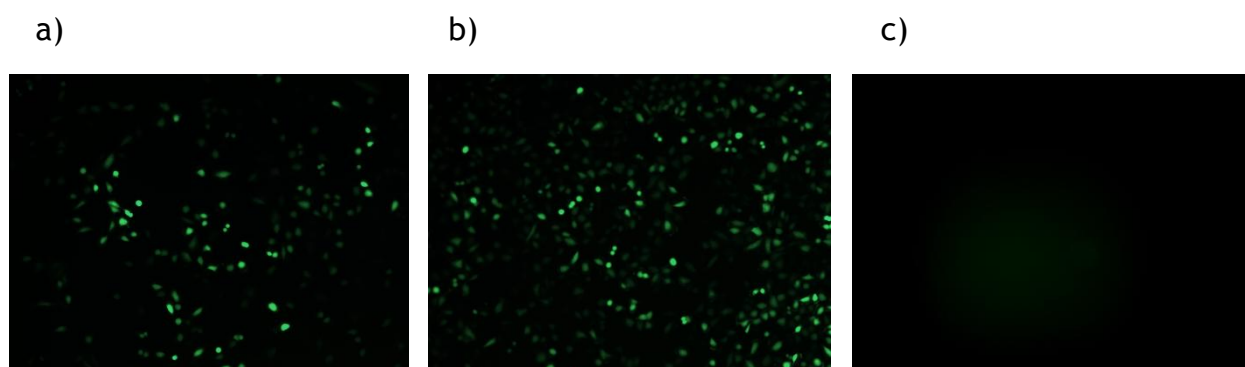


Figure 3.3 CRFK cells transduced with rabies virus, VSV-G or Δ Env bearing MLV pseudotypes. CRFK cells were transduced with a) MLV(rabies) pseudotypes b) MLV(VSV) pseudotypes or c) MLV(Δ Env) negative control pseudotypes and analysed by fluorescence microscopy at 48 h.

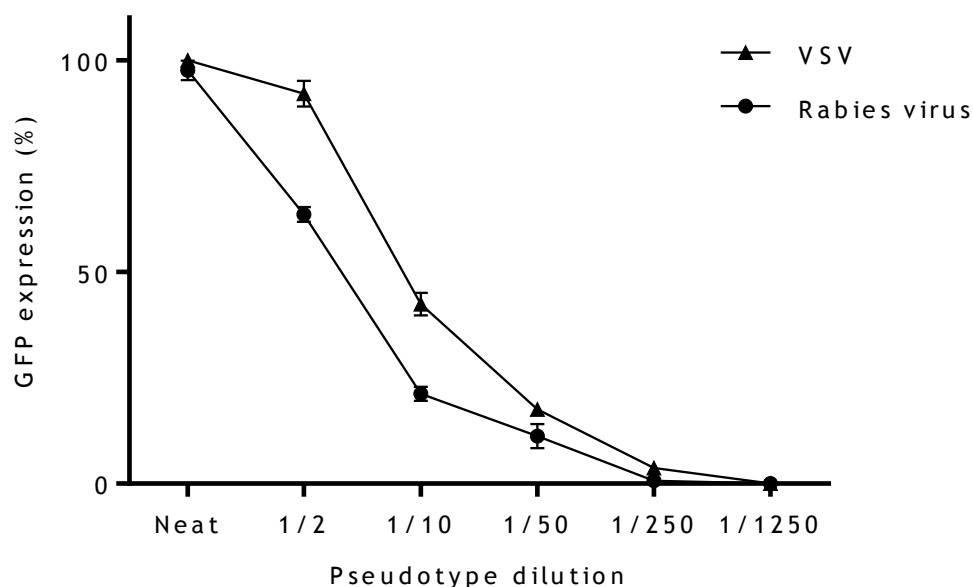


Figure 3.4 Serial dilution of MLV(rabies) and MLV(VSV) pseudotypes transduced into CRFK cells. Five-fold serial dilutions of each pseudotype were titrated onto CRFK cells and analysed by flow cytometry at 48 h post transduction.

To determine the most effective target cell line for use in our neutralisation test, a variety of cell lines, both adherent (BHK-21, Vero, CRFK, D17) and suspension (MT-4, BMOX40, 3201, SUP-T1), were used for transduction of MLV(rabies) and MLV(VSV) pseudotypes. The results demonstrate that the proportion of GFP expressing cells varied with the target cell line. MLV(rabies) pseudotypes infected all of the adherent cell lines, whereas the suspension cell lines appear to be refractory to infection with MLV(rabies) pseudotypes (Figure 3.5). BHK-21 cells are routinely used for the FAVN and were initially the first choice of target cell line for the pNT, however BHK-21 cells showed a low level of GFP expression (3.6%) and were therefore discounted for use in the pNT. Vero, CRFK and D17 cell lines expressed similar levels of GFP when transduced with MLV(rabies) pseudotypes and although D17 cell lines displayed the highest level of GFP expression, this cell line was discounted as a target cell line for use in the pNT as the origin of our stock of D17 cells could not be verified. Whilst

GFP expression in Vero cells was high by flow cytometry, it proved difficult to interpret by fluorescence microscopy. Some of the transduced Vero cells expressed GFP at a low level and cell boundaries could be difficult to distinguish (Figure 3.6). CRFK cells were chosen as the target cell line for the pNT as we had a validated stock of cells sourced from the American Type Culture Collection, GFP expression was of a high intensity and individually fluorescing cells were easily identifiable by fluorescence microscopy. The results of our cell line infectivity experiment show a decreased titre of MLV(rabies) compared to MLV(VSV) pseudotypes. However it is important to note that the concentrations of the input pseudotype were not matched (only volume was matched), therefore the difference may be due to a higher concentration of MLV(VSV) pseudotypes being produced following transfection rather than an increased ability for MLV(VSV) pseudotypes to mediate cell entry.

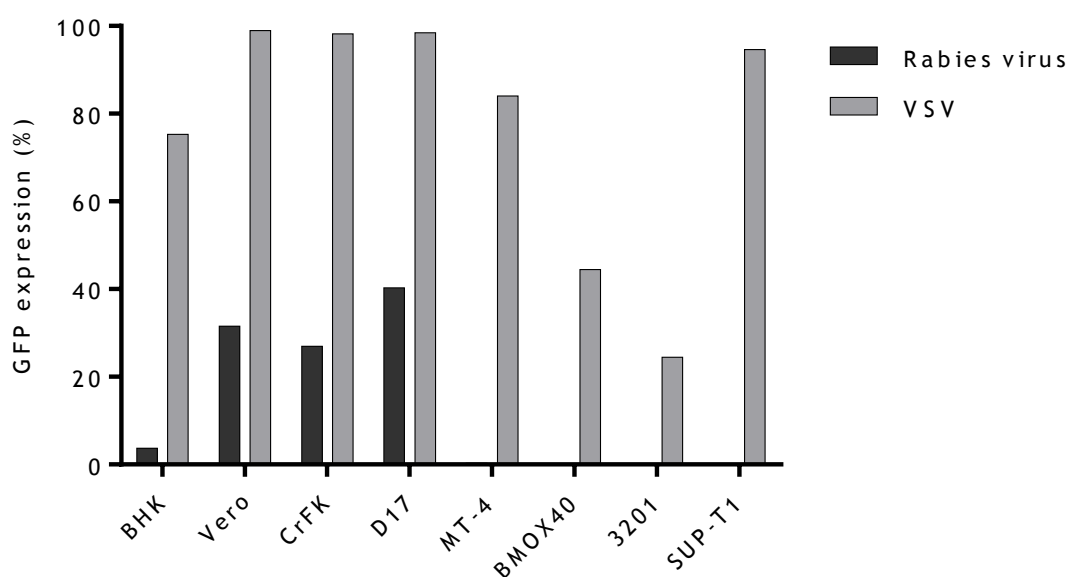


Figure 3.5 Ability of pseudotypes bearing rabies virus or VSV glycoproteins to transduce adherent and suspension cell lines. MLV(rabies) and MLV(VSV) pseudotypes were transduced into the adherent cell lines; BHK-21, Vero, CRFK and D17, and the suspension cell lines; MT-4, BMOX40, 3201, SUP-T1, and GFP expression measured by flow cytometry at 48 h post transduction. Assay was performed in 96-well plates with 100 μ l of pseudotype added (n=1).

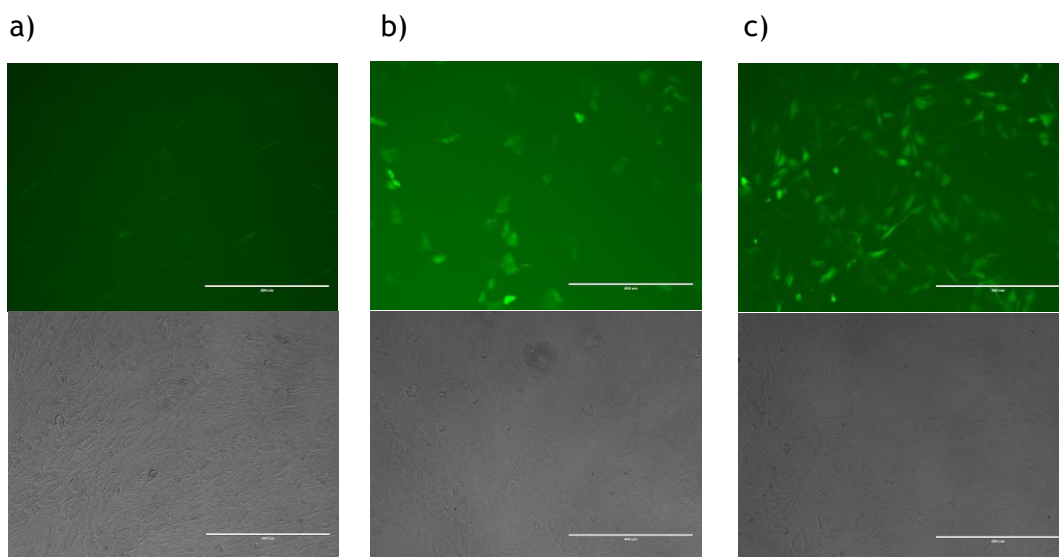


Figure 3.6 Fluorescence and phase contrast micrographs of MLV(rabies) pseudotypes transduced into three potential target cell lines. GFP expression following transduction with MLV(rabies) pseudotypes was observed using fluorescence microscopy at 48 h post transduction in a) BHK-21 b) Vero and c) CRFK cell lines. Line represents scale bar (400 μ m).

To determine the optimum incubation time for infection with viral pseudotypes in our pNT, CRFK cells were transduced with MLV(rabies) and MLV(VSV) pseudotypes in duplicate. One well was analysed by flow cytometry at 48 h and the other at 72 h. There was a slight, but non-significant, decrease in GFP expression at 72 h compared to 48 h in cells transduced with either MLV(rabies) or MLV(VSV) pseudotypes (Figure 3.7). As the expression levels were similar, and to decrease the turn-around time of the assay, an incubation time of 48 h was chosen for neutralisation assays.

To investigate the effect of freeze thawing on our MLV(rabies) pseudotypes, pseudotypes were exposed to 15 freeze-thaw cycles and GFP expression measured by flow cytometry. The results demonstrated a significant decrease in titre of the MLV(rabies) pseudotypes, with a greater than 50% decrease in pseudotype titre by the fifth freeze-thaw cycle (Figure 3.8). MLV(VSV) pseudotypes were more stable than MLV(rabies) pseudotypes, with an average

loss in pseudotype titre of 2.15% per freeze thaw cycle compared to 6.67% observed with MLV(rabies) pseudotypes. As the rabies virus pNT will be based on a specific, low input pseudotype concentration, small changes in titre will have a dramatic effect on test accuracy. Therefore all neutralisation tests were performed using aliquots of pseudotypes that have been thawed only once.

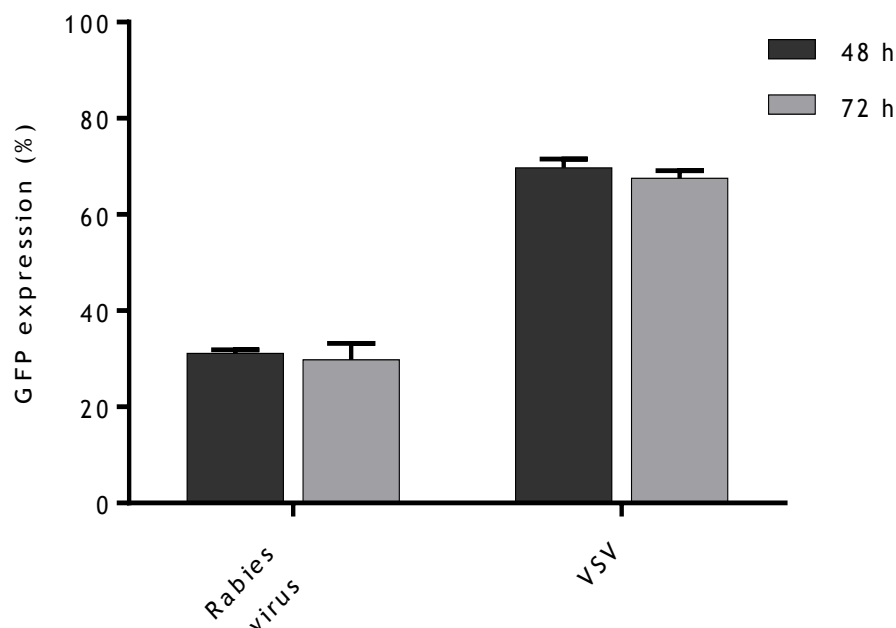


Figure 3.7 Expression of GFP in CRFK cells transduced with MLV(rabies) and MLV(VSV) pseudotypes for 48 h and 72 h. CRFK cells were infected with MLV(rabies) or MLV(VSV) pseudotypes and GFP expression was measured at 48 h and 72 h post transduction by flow cytometry. Assay was performed in 12 well plates with 500 μ l of pseudotype added. Each bar represents the mean \pm standard error (n=3).

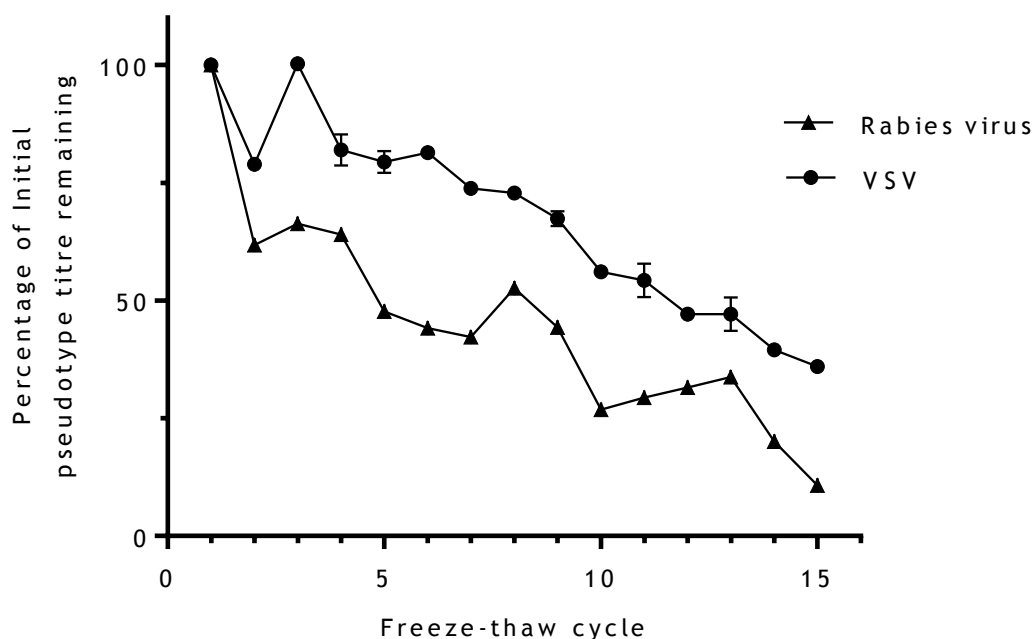


Figure 3.8 Effect of multiple cycles of freeze-thawing on the stability of MLV(rabies) and MLV(VSV) pseudotypes. MLV pseudotypes bearing the glycoprotein of rabies virus or VSV were exposed to multiple freeze-thaw cycles prior to transduction of CRFK cells. The percentage of the initial pseudotype titre remaining was measured by flow cytometry at 48 h. Measurements were performed in triplicate; each point represents the mean \pm standard error.

3.2.2 Development and validation of a rabies virus pseudotype neutralisation test

In this study we used the MLV(rabies) pseudotypes described in 3.2.1 in a pNT for the detection of VNAb against rabies virus in serum samples. To facilitate inter-test comparisons, our pNT was broadly based on protocols used in the gold standard FAVN test. Briefly, in the FAVN test serum samples are serially diluted three-fold and added to four wells of a 96-well plate. A fixed dose of rabies virus is then added to each well and incubated for 1 h at 37°C. Following incubation, BHK-21 cells are added to each well and incubated for a further 48 h. Plates are then washed in PBS, fixed in acetone, stained with a FITC-conjugated anti-rabies antibody and incubated for 30 min. Plates are again washed in PBS and read by

fluorescence microscopy. A well is considered positive if there are one or more fluorescent cells per well. A titre is determined as the highest dilution at which 100% of virus is neutralised in at least 50% of the wells (Cliquet et al., 1998). In addition to replacing live rabies virus with MLV-based rabies virus pseudotypes, our assay was further modified from the FAVN. Briefly, in the pNT cells were seeded 18 h prior to commencing the assay, serum samples are serially diluted two-fold and incubated with a fixed dose of pseudotype for 1 h at 37°C. The serum/pseudotype mix is then added directly to three wells of a 96-well plate. After 48 h incubation plates are read directly, no washing of cells is required and removal of the culture medium is not necessary. As in the FAVN, a well is considered positive (and therefore not neutralised) if one or more fluorescent cells are present. A titre is reported as a reciprocal of the highest dilution at which 100% of virus is neutralised in at least two of the three wells. By using three wells per sample instead of four, the volume of serum required per test is decreased and further dilutions of sera can be analysed. Additionally each serum is tested against VSV-G bearing pseudotypes at the lowest serum dilutions, this control is included in the assay to detect non-specific neutralisation, as there have been reports of non-specific neutralisation, toxicity and inhibition in the FAVN and RFFIT (Shiraishi et al., 2014a, Bedeković et al., 2013, Mani and Madhusudana, 2013). If the serum neutralises the MLV(VSV) pseudotypes at a titre of >20, the sample is repeat tested against MLV(VSV) pseudotypes with further serum dilutions of up to 1:640, to determine if the observed neutralisation is specific. The VSV control is also useful for detecting cytotoxicity, as MLV(VSV) pseudotypes cannot infect dead cells and therefore the wells would be negative for GFP expression, which could be misinterpreted as rabies VNAb specific neutralisation to an inexperienced user in the absence of the VSV control. A rabies VNAb positive and negative control canine serum is added to one plate per run. A schematic diagram of a plate map for the rabies pNT is shown in Figure 3.9. To calculate a working concentration of pseudotype for use in the pNT, the TCID₅₀ was calculated for the MLV(rabies) and MLV(VSV) pseudotypes. A fixed pseudotype concentration of 100 TCID₅₀ was added per well to mirror the titre of live virus used in the FAVN.

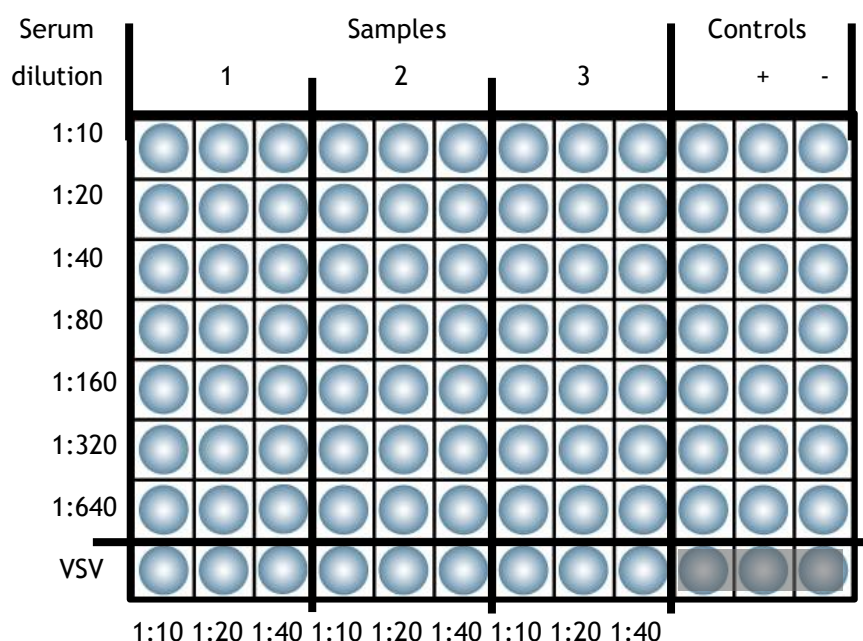


Figure 3.9 Schematic representation of the plate set up for MLV(rabies) pseudotype neutralisation test. Each test sample is serially diluted two-fold from 1:10 to 1:640 and tested in triplicate. Each test serum is also tested against MLV(VSV) pseudotypes at serum dilutions of 1:10 to 1:40 as a measure of non-specific neutralisation. Rabies antibody positive and negative sera are also added to one plate per assay run as test controls.

To validate this assay serum samples which had previously been tested for rabies VNAb by FAVN were tested using the rabies virus pNT. These samples had also previously been tested using another pseudotype-based neutralisation assay, this pNT was performed using a lentiviral pseudotype bearing a luciferase reporter gene (HIV(luc) pNT) (Wright et al., 2009). The serum samples tested were from domestic dogs living within the Serengeti district of Tanzania. The dogs had been enrolled in an annual vaccination campaign and were kindly provided by the Viral Transmission Dynamics Project (Table 3.1).

		Age				Total
		2-6 m	7-12 m	1-4 y	5-8 y	
Sex	Male	12	8	20	6	46 (44%)
	Female	18	13	21	6	58 (56%)
Total		30 (29%)	21 (20%)	41 (39%)	12 (12%)	104 (100%)

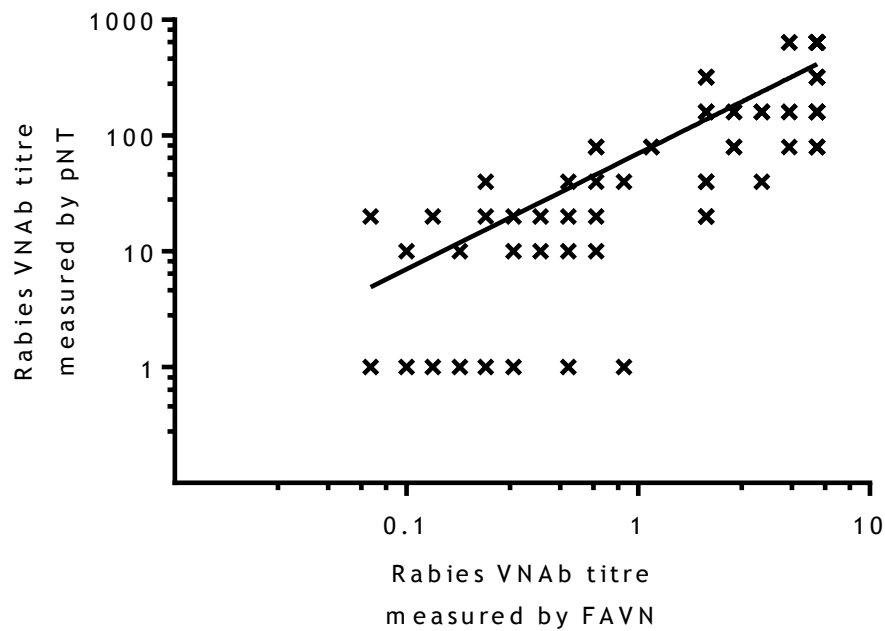
Table 3.1 Sex and age data of canine sera tested by rabies virus pseudotype neutralisation test.

The result of the FAVN is converted from a dilution factor to IU/ml, the VNAb titre of both pNTs is reported as the reciprocal of the dilution factor. Samples that failed to neutralise the pseudotype at the lowest dilution, 1:10, were given a titre of <10 and assigned an arbitrary titre of 1 for data analysis. Of the 104 serum samples tested on the rabies virus pNT, one sample failed the initial VSV control (#71). This sample was additionally tested against VSV with further serum dilutions and a VSV VNAb titre of 640 was recorded (alongside a rabies VNAb titre of 320). Given that the cell monolayer looked healthy and confluent, we concluded that this result was most likely due to non-specific neutralisation or inhibition. This reinforces the requirement of adequate controls as the same sample tested by FAVN had a titre of 0.13 IU/ml (considered a non-protective rabies VNAb titre), without the VSV control the result of the rabies pNT would have been misinterpreted as rabies VNAb positive.

Our results demonstrate that as the rabies VNAb titre increases in the FAVN test, the titre recorded by our pNT also increases (Figure 3.10). The correlation between our pNT and the FAVN test and HIV(luc) pNT was 0.776 and 0.833 respectively ($p < 0.0001$). In order to assess the robustness of our assay we repeated a small number ($n=12$) of serum samples using the same method utilising a new batch of MLV(rabies) pseudotypes and using Vero cells as the

target cell line. The high correlation between VNAb titres seen by both assays ($R^2=0.904$, $p<0.0001$) demonstrates the reproducibility of the assay (Figure 3.11).

a)



b)

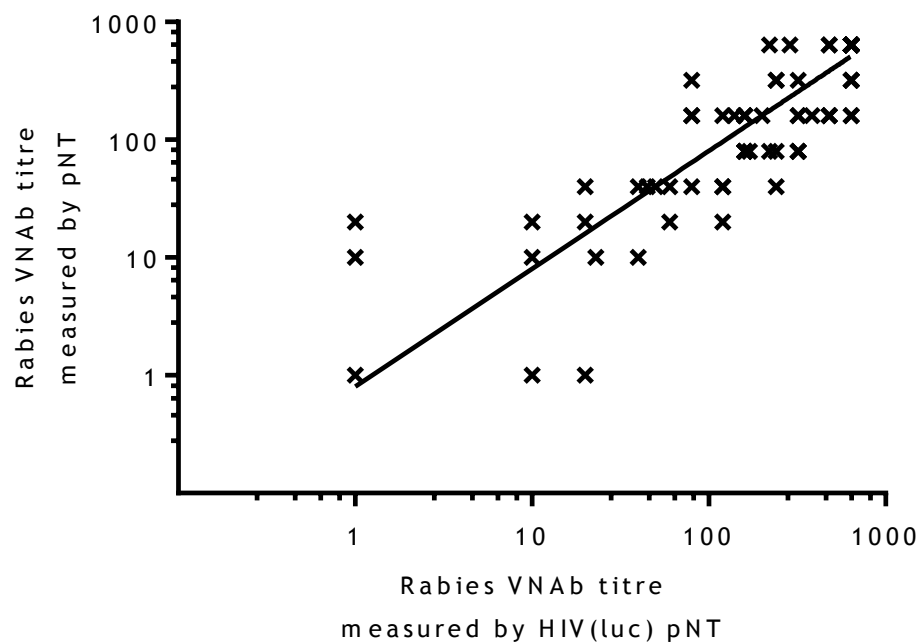


Figure 3.10 Correlation of rabies VNAb antibody titres determined by the rabies virus pNT, the FAVN test and the HIV(luc) pNT. Rabies VNAb titres obtained by the rabies virus pNT were compared with a) the FAVN test (n=103, $R^2=0.603$, $p<0.0001$) and b) the HIV(luc) pNT (n=103, $R^2=0.695$, $p<0.0001$). Correlation and p-values were calculated using Pearson's product-moment correlation.

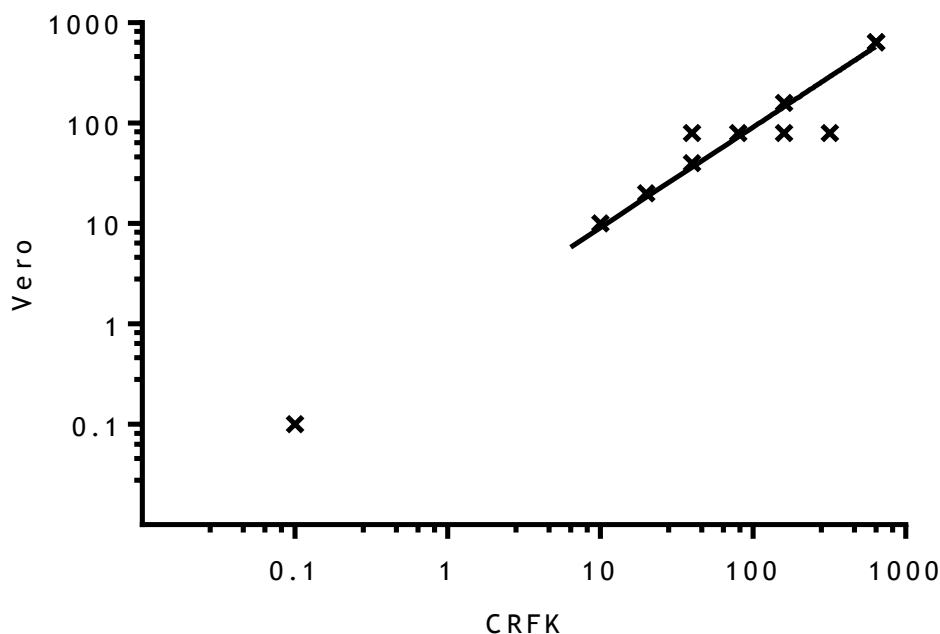


Figure 3.11 Correlation of rabies VNAb titres obtained using either CRFK or Vero cells as the target cell line for the rabies virus pseudotype neutralisation test. A new batch of MLV(rabies) pseudotypes were produced, TCID₅₀ calculated and the pNT performed on Vero cells. Rabies VNAb titres were compared to titres previously measured on the pNT using CRFK cells. A high degree of correlation of VNAb titre was observed between the assays (n=12, $R^2=0.904$, $p<0.0001$). Correlation and p-values were calculated using Pearson's product-moment correlation.

At the time of testing the canine sera, we were unable to obtain the OIE reference serum of dog origin, the international standard used in the FAVN for converting rabies VNAb titres in IU/ml. This reference serum is diluted to a titre of 0.5 IU/ml, the minimum antibody titre considered to give protection against rabies virus challenge (WHO, 2010). Ideally, as in the FAVN, this sample would have been tested alongside all test sera to determine an accurate rabies VNAb titre in IU/ml. After testing these samples we were able to source a small volume of this reference serum which was then tested using our rabies virus pNT. The serum was tested in triplicate on three separate days, to allow for inter-assay variation, and at each test the titre of the sample was 40. We

therefore used a neutralising titre of 40 in our rabies virus pNT to correlate to 0.5 IU/ml. Using this cut-off we were then able to calculate the false positive and false negative rates in our assay. Positivity was classified as a VNAb titre of ≥ 0.5 IU/ml by FAVN or ≥ 40 by rabies virus pNT.

Of the 104 serum samples tested, one was inconclusive due to failing the VSV control. There were 9 discordant samples among the remaining 103 samples, 8 of which tested positive by the FAVN and negative by rabies virus pNT and one of which tested negative by the FAVN and positive by rabies pNT (Table 3.2). The rate of false negatives compared to the gold standard FAVN was 0.077 (n=8) and the rate of false positives was 0.001 (n=1) (Figure 3.12). When our results were compared with the HIV(luc) pNT the false negative and positive rates were 0.029 (n=3) and 0.001 (n=1) respectively. The majority of the discordant results clustered around the assay cut-off titre (0.5 IU/ml), with 63% of the false negative results being assigned to the two lowest FAVN titres considered positive (0.5 and 0.66 IU/ml). Although these false negatives decreased the sensitivity of the test to 81.82% (95% CI: 67.28 % to 91.78 %), the specificity remained high at 98.21% (95% CI: 90.88 % to 99.72 %).

Sample number	Age	Sex	Previous vaccination	FAVN test	HIV(luc) pNT	Rabies virus pNT
69	9m	F	N	0.66	20	20
75	3y	F	Y	0.5	<10	<10
94	2y	F	Y	0.5	<10	10
102	7m	F	N	0.87	10	<10
121	1y	F	N	0.22	20	40
171	6m	M	N	0.5	<10	20
212	4y	F	Y	1.97	40-80	20
314	5m	F	Y	1.97	80-160	20
381	2y	M	Y	0.66	40	10

Table 3.2 Details of serum samples for which discordant results were observed between the rabies virus pNT and the FAVN test. Age, sex and vaccination history of dogs whose VNAb response by the rabies virus pNT was discordant with results of the FAVN test. Results of rabies VNAb titres measured by the HIV(luc) pNT are also included.

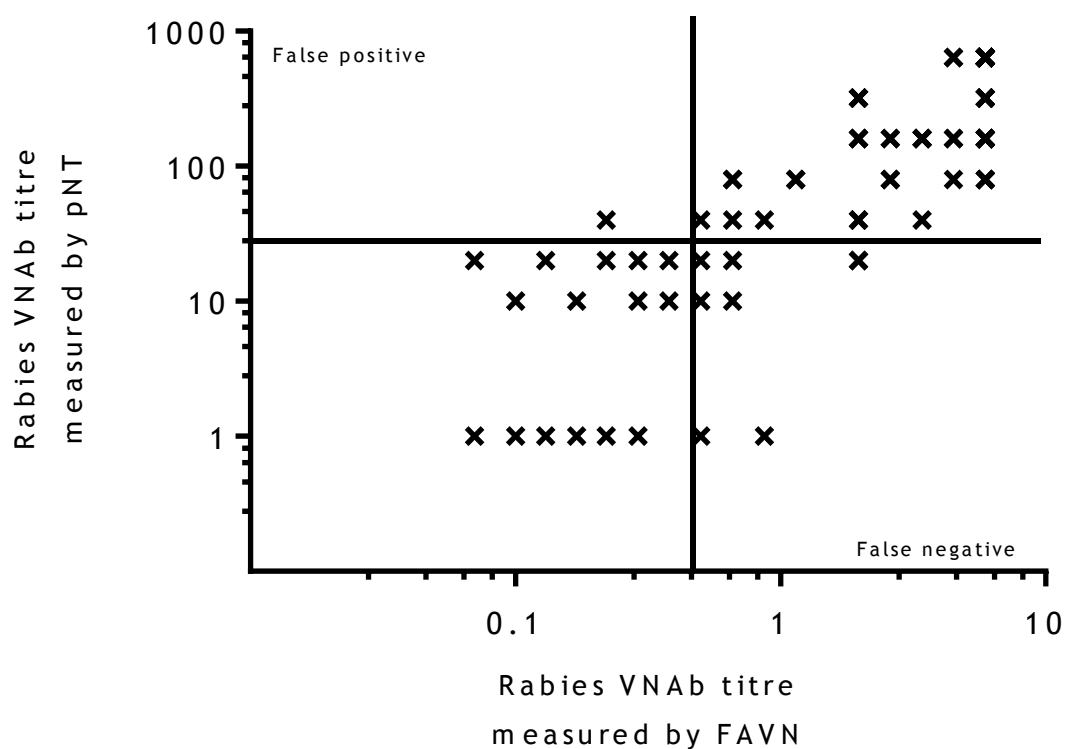


Figure 3.12 False positive and false negative rates between the rabies virus pseudotype neutralisation test and the FAVN test. When samples were classified as positive or negative by VNAb titre (≥ 0.5 IU/ml by FAVN and ≥ 40 by rabies virus pNT) there was a false negative rate of 0.077 and a false positive rate of 0.001 between the rabies virus pNT and the FAVN test (n=103).

3.2.3 Optimisation of the rabies virus pseudotype neutralisation test

The volume of OIE reference standard we were able to obtain was limited, therefore we had no reference standard to run on future rabies virus pNT. To mimic the OIE reference serum, which consistently gave a VNAb titre of 40 on the rabies virus pNT, a human serum sample from a vaccinated individual was diluted to give a titre of 40 on the rabies virus pNT. This human standard was subsequently included in all future tests along with positive and negative canine serum controls, if this standard did not give a titre of 40, the test samples were repeated, as a cut-off for adequate protective antibody could not be extrapolated.

A volume of 90 µl of serum is required for the pNT to allow for serial dilutions of sera in triplicate, VSV controls and an additional 10% to allow for pipetting error. A volume of 50 µl of diluted serum and 50 µl of pseudotype was initially used in the pNT. Due to the potential for small volumes of serum to be received for testing on the rabies virus pNT, the volumes used in the assay were halved decreasing the volume of serum required for testing to 45 µl. To determine whether the lower volumes would affect the reproducibility of the test, 19 canine serum samples, in addition to the three test controls, were tested in duplicate using both the original and half volumes of sera. The rabies VNAb titres were identical for all samples in each test resulting in a correlation of 100% (data not shown). For all future pNT these lower volumes were implemented, resulting in an input dose of 50 TCID₅₀.

3.2.4 Pseudotype-based neutralisation tests for non-rabies lyssaviruses

After successfully establishing a protocol for the rabies virus pNT, we developed a pNT for measuring VNAb to other members of the *Lyssavirus* genus. Plasmids encoding 6 other lyssavirus glycoproteins were kindly provided by Dr. Edward Wright (University of Westminster). Pseudotypes were produced as previously described with substitution of the CVS-11 plasmid with that of another lyssavirus glycoprotein. There are 14 species of lyssavirus, a pNT for rabies and 6 of the other major species were developed in this study; Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type-1 (EBLV-1), European bat lyssavirus type-2 (EBLV-2) and Australian bat lyssavirus (ABLV). Lyssavirus species fall into two distinct phylogroups (Badrane et al., 2001),

phylogroup 1 contains rabies virus, DUVV, EBLV-1, EBLV-2 and ABLV, and phylogroup 2 contains LBV and MOKV (Figure 3.13). TCID₅₀ was calculated for each MLV(lyssavirus) pseudotype and CRFK cells were infected with matched concentrations of each of the 7 MLV(lyssavirus) pseudotypes to determine if this cell line was permissive to all MLV(lyssavirus) pseudotypes. In all cases GFP was detectable by flow cytometry and fluorescent microscopy (Figure 3.14).

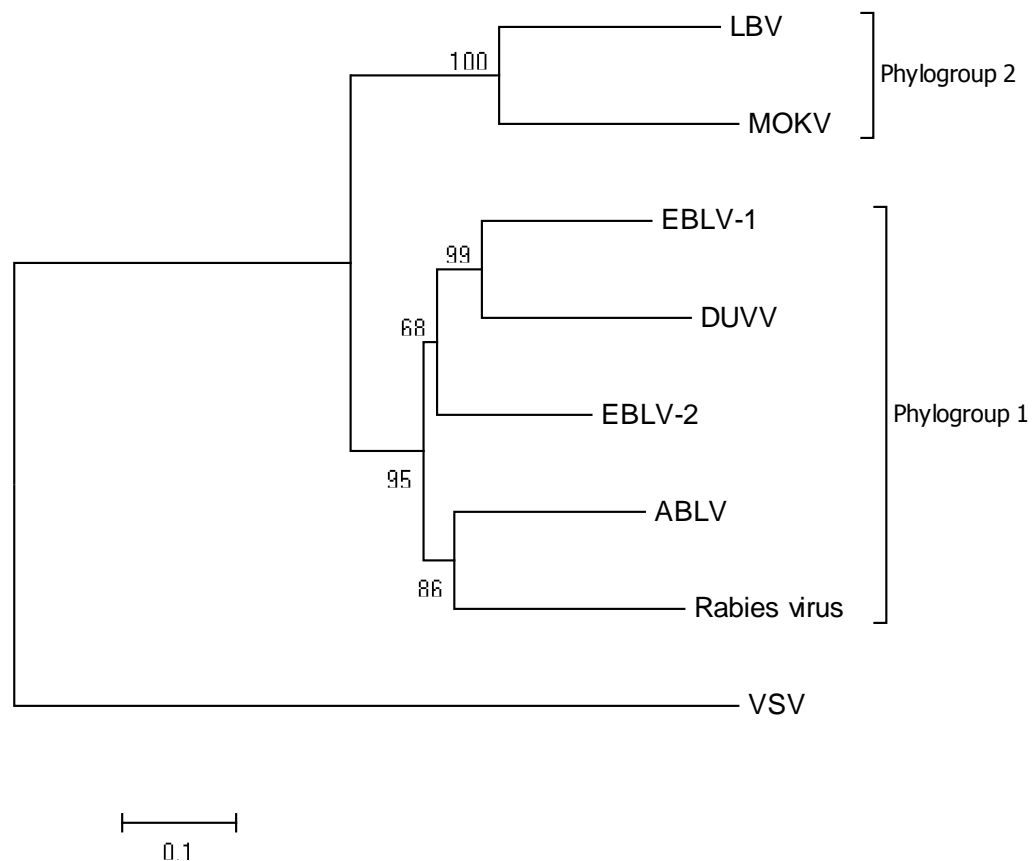
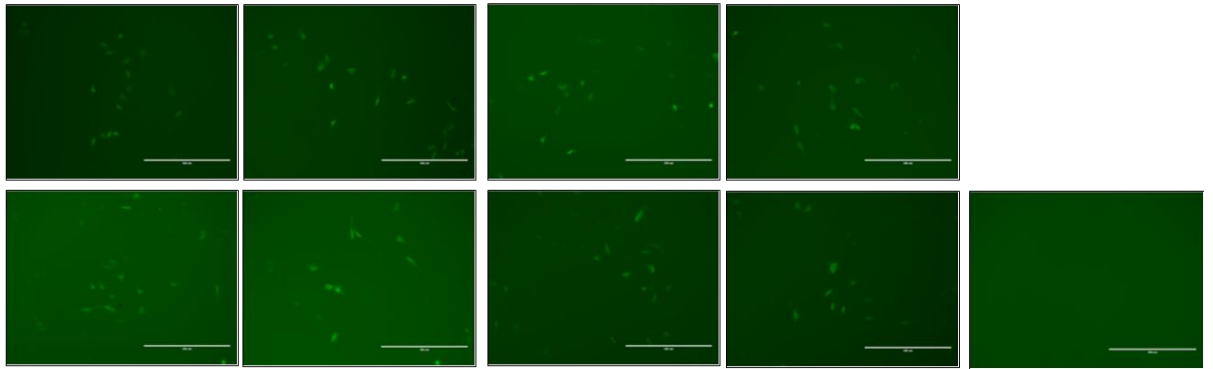


Figure 3.13 Phylogenetic analysis of the seven lyssavirus species investigated in this study. The full length lyssavirus glycoprotein gene sequences used to produce viral pseudotypes in this study were used to construct a phylogenetic tree. The tree was determined by the neighbour-joining method, where branch length is proportional to evolutionary distances between lyssaviruses. The numbers at each node represent the percentage bootstrap values (1000 replicates). Evolutionary analysis was conducted in MEGA6 (Tamura et al., 2013).

a)



b)

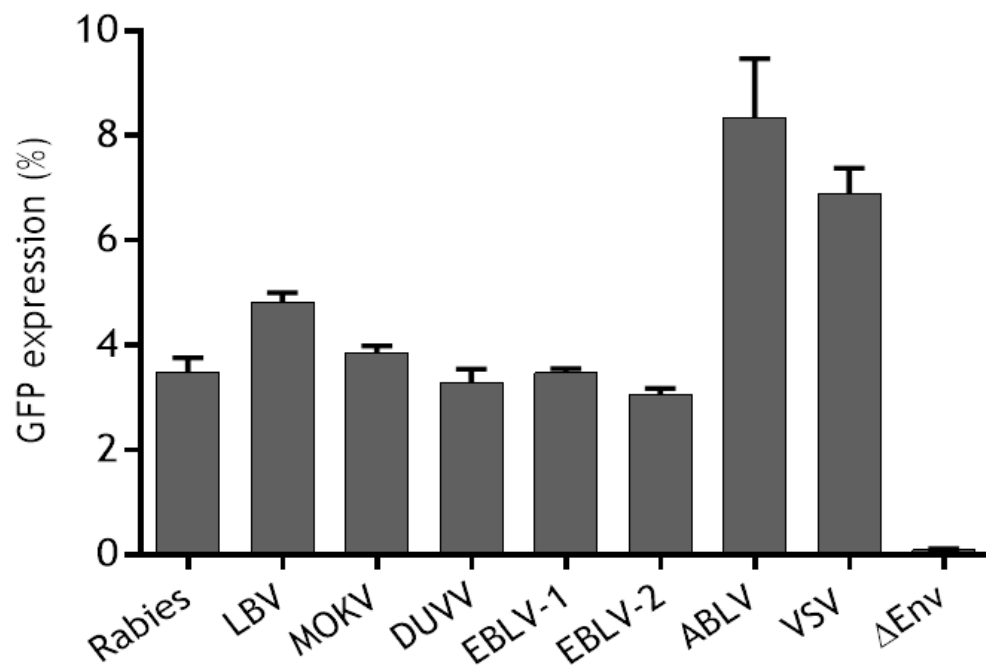
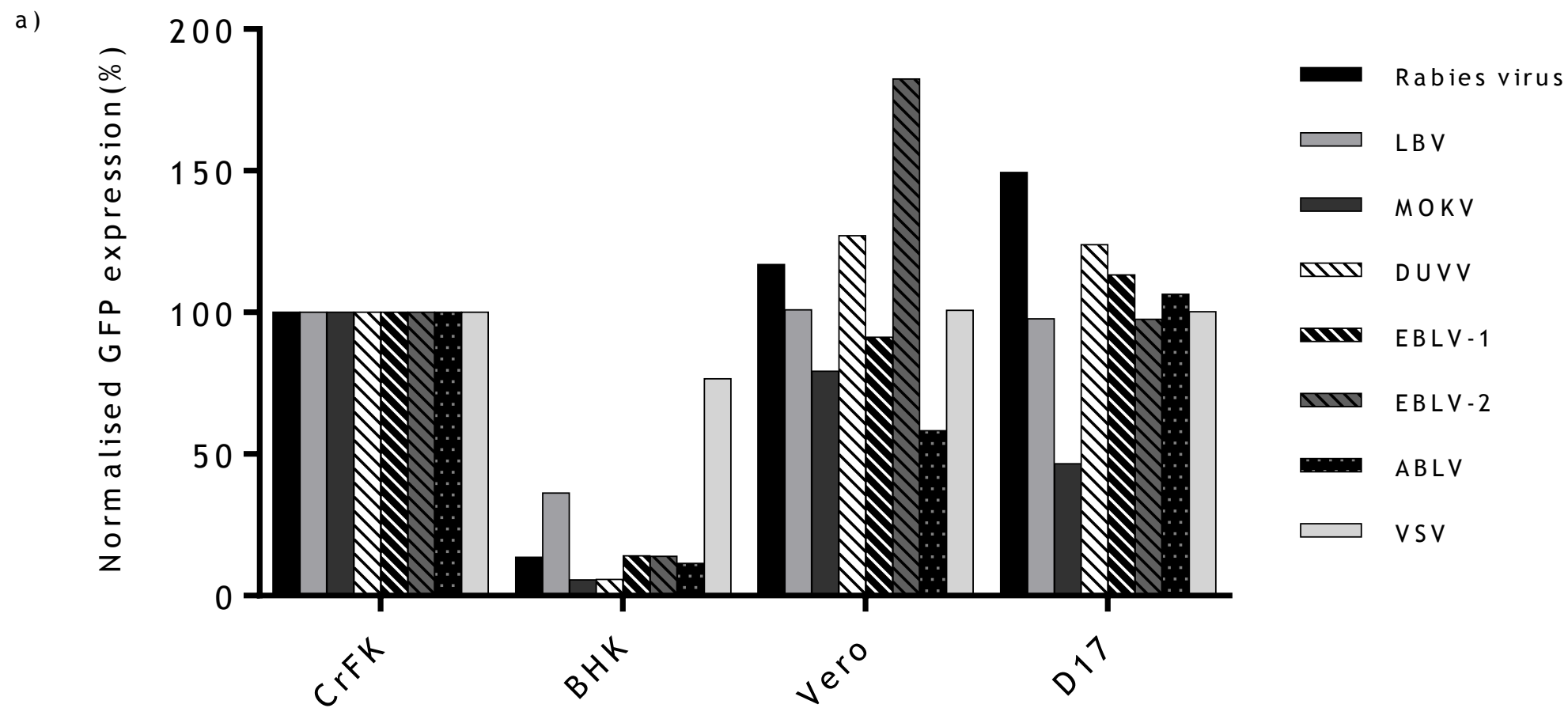


Figure 3.14 Transduction of CRFK cells with MLV-based pseudotypes bearing lyssaviral glycoproteins. 1000 TCID₅₀ of each MLV(lyssavirus) pseudotype was incubated with CRFK cells and GFP expression was assessed at 48 h by a) fluorescence microscopy (pictures are displayed in the same order as depicted in the graph). The line represents scale bar (400 μ m), and b) flow cytometry, each bar represents the mean \pm standard error (n=3).

The percentage of target cells expressing GFP following transduction was approximately 4% for the majority of MLV(lyssavirus) pseudotypes, although expression seen in cells transduced with MLV(ABLV) and MLV(VSV) pseudotypes were twice this level, suggesting that their TCID₅₀ calculation may have been incorrect. The TCID₅₀ was repeated with both pseudotypes and found to be the same as the original value, although the results by flow cytometry were higher (Figure 3.14b), the number of GFP expressing cells observed by fluorescence microscopy was similar to that seen with the other lyssaviruses (Figure 3.14a).

We previously demonstrated that the infectivity of MLV(rabies) pseudotypes was dependent on target cell line (Figure 3.5). To investigate the permissiveness of cell lines to the other lyssaviral pseudotypes, we infected 8 cell lines with 100 µl of each MLV(lyssavirus) pseudotype. As the concentration of the inoculum was not matched, the GFP expression was normalised against the expression level observed in CRFK cells. The infectivity profiles seen in the adherent cell lines (CRFK, BHK-21, Vero, D17) were similar for all MLV(lyssavirus) pseudotypes, with BHK-21 being least permissive and similar expression levels demonstrated in CRFK, Vero and D17 cells. The expression levels observed in suspension cell lines differed considerably between the lyssaviral pseudotypes. MLV(rabies) pseudotypes infected only MT-4 cell lines and at less than 1%. All other MLV(lyssavirus) pseudotypes infected both MT-4 and SUP-T1 cells, both T cell lymphoblast cell lines, with MLV(DUVV) and MLV(LBV) exhibiting the highest titres. In BMOX40 and 3201 cell lines, MLV(DUVV) was the only pseudotype to infect these cells at greater than 1% (Figure 3.15).



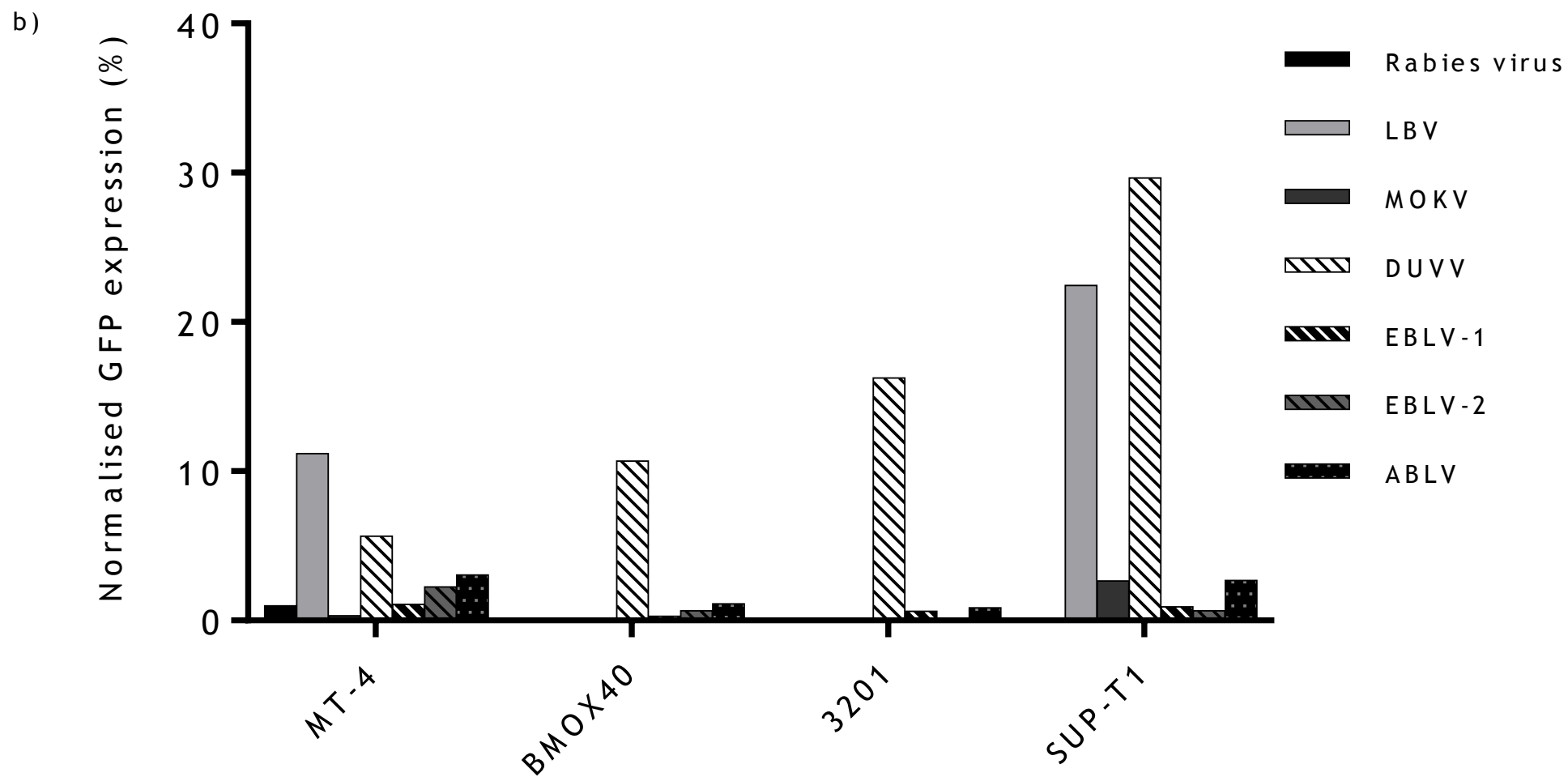


Figure 3.15 Expression of GFP in target cell lines following transduction with MLV(lyssavirus) pseudotypes. Pseudotypes were transduced into eight cell lines a) four adherent cell lines (BHK-21, CRFK, Vero, D17) and b) four suspension cell lines (MT-4, BMOX40, 3201, SUP-T1). GFP expression was normalised against the expression levels measured in CRFK cells, as the pseudotype concentrations were not matched.

With high titre pseudotypes for seven species of lyssavirus, we were keen to test their use in a pNT, however unfortunately VNAb positive serum to these lyssaviruses is uncommon. As we were unable to obtain any neutralising sera to these lyssaviruses, we used the previously tested Tanzanian dog sera to test against pseudotypes bearing the LBV, MOKV and DUVV glycoproteins in the pNT. Rabies VNAb have been shown to cross-neutralise other lyssaviruses from phylogroup 1 but do not cross-neutralise viruses from phylogroup 2 (Lafon et al., 1988, Brookes et al., 2005b, Hanlon et al., 2005). We compared rabies VNAb titres measured by the rabies virus pNT to VNAb titres of DUVV (phylogroup 1) and LBV and MOKV (phylogroup 2) (Figure 3.16). There was a significant positive correlation in VNAb titres observed in the rabies virus and DUVV pNT ($R^2=0.388$, $p<0.0001$), as would be expected as DUVV is a phylogroup 1 lyssavirus. However there was no correlation observed between rabies VNAb titres and either of the phylogroup 2 lyssaviruses (LBV $R^2=0.018$ $p=0.484$, MOKV $R^2=0.007$ $p=0.6704$). Correlation and p-values were calculated using Pearson's product-moment correlation. There is currently no recommended titre considered to provide adequate protection to challenge for any of the lyssaviruses other than rabies virus, but if we use the same cut-off as for rabies virus (titre ≥ 40) none of the samples tested were considered cross-neutralising to either of the phylogroup 2 lyssaviruses, even from samples demonstrating the highest rabies VNAb titre.

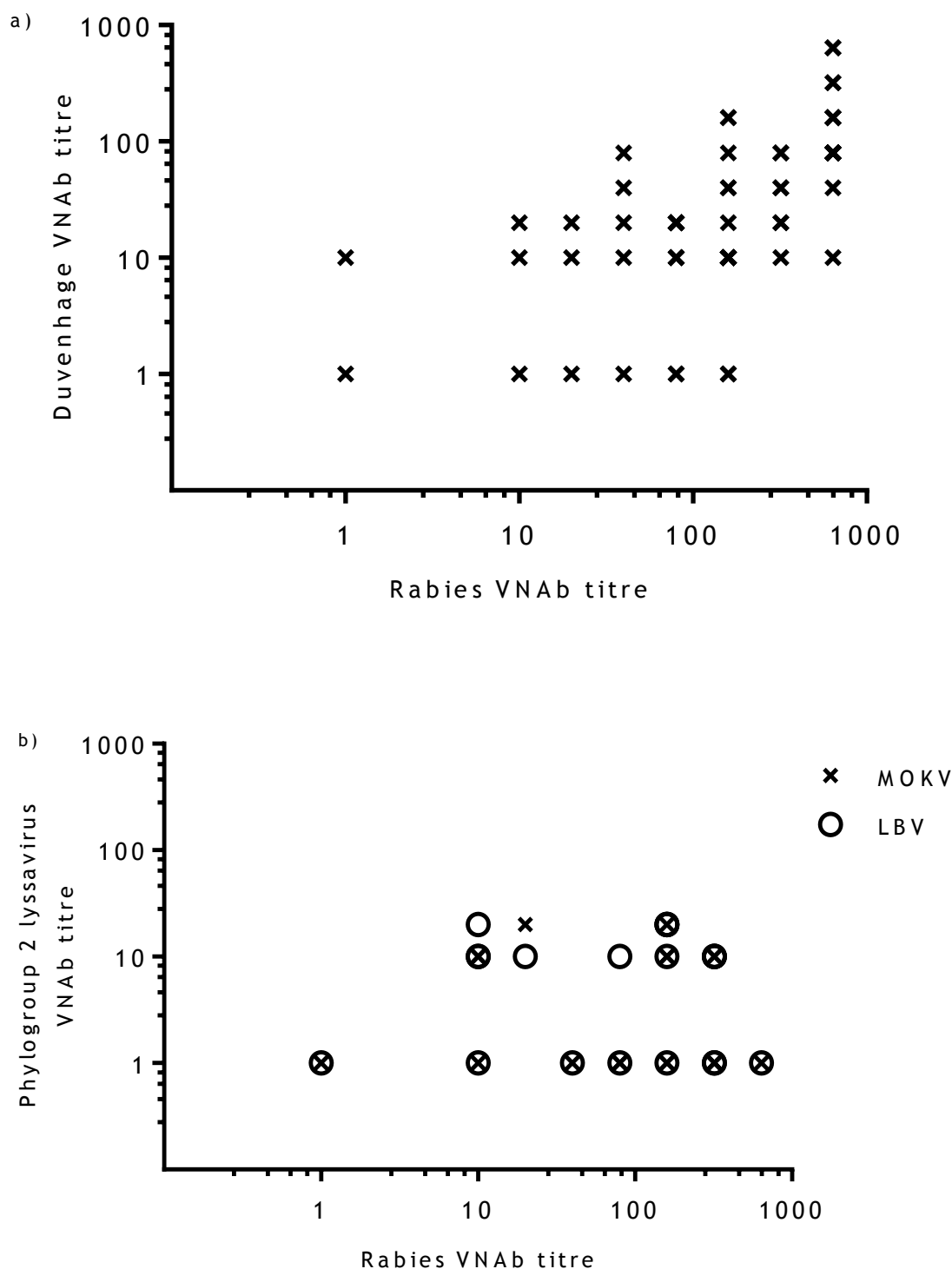


Figure 3.16 Cross-neutralisation of lyssavirus pseudotypes by sera raised against rabies virus. Serum samples were tested by pNT using a) MLV(rabies) and MLV(DUVV) pseudotypes ($n=103$, $R^2=0.388$, $p<0.0001$) and b) MLV(LBV) ($n=30$, $R^2=0.018$, $p=0.4844$) and MLV(MOKV) pseudotypes. ($n=30$, $R^2=0.007$, $p=0.6704$). These pNT were performed prior to the change in volumes mentioned in section 3.2.3 and were therefore tested with 50 μ l of test sera and 100 TCID₅₀ of pseudotype.

3.3 Discussion

The development of reliable, accessible and economical assays for rabies virus serosurveillance will contribute to improving rabies prevention and control strategies (OIE, 2008). This study demonstrates that pseudotypes bearing the glycoprotein of lyssaviruses can be used successfully as a substitute for live virus in neutralisation tests. Handling lyssaviruses requires a SAPO licence and the requirements for work involving lyssaviruses is particularly stringent (DEFRA, 2015), therefore currently rabies VNAb testing is limited to the few laboratories where adequate containment facilities exist. The rabies virus pNT negates the requirement for high containment facilities, which are often unavailable in countries where rabies is still endemic. Additionally the cost of rabies VNAb testing is prohibitive for many serosurveillance studies, with the price of one FAVN test costing £45 (Biobest, 2015). In contrast, the cost of consumables for the pNT developed in this study is less than £1 per sample.

The flexibility of the assay also allows for the measurement of VNAb to any of the lyssaviruses by simply replacing the rabies virus glycoprotein with the glycoprotein of the chosen lyssavirus. Therefore, if a new lyssavirus is identified, only the sequence of the glycoprotein is required to set up a pNT, whereas in the FAVN the virus needs to be isolated and cultured to a high titre, which can prove time consuming. The requirement for robust assays for determining exposure to novel lyssaviruses has become more pressing, with 5 of the 14 species of lyssavirus isolated within the last 14 years (Marston et al., 2012, Kuzmin et al., 2010, Freuling et al., 2011, Botvinkin et al., 2003). Flexibility of the assay is also further improved by easy modification of the reporter gene utilised in the pNT, which can be changed depending on the preference of the laboratory, previous rabies pNTs have also been described using both luciferase and *lacZ* (Wright et al., 2008). The preference for utilising GFP as the reporter gene in this study was determined by the lack of post-assay processing required and the lower cost of fluorescence microscopy compared to luciferase assay development. The drawback of using GFP as the reporter is that the assay needs to be read by fluorescence microscopy and the entire area of each well must be screened as the presence of one fluorescent cell is significant. This manual screening not only increases the time taken to read the assay but also leaves some subjectivity in assay interpretation. However the assay could be optimised

for analysis by flow cytometry if required, which would be especially beneficial if a suitable suspension cell line could be obtained.

The MLV-based pseudotype system was shown to give a low reporter background in this study as no non-specific fluorescence was demonstrated in cells transduced with MLV(Δ Env) pseudotypes. The disadvantage of the MLV pseudotypes, evidenced in this study, is that they do not appear to be as stable through successive cycles of freeze-thawing, as other pseudotype systems based on HIV and VSV (Molesti et al., 2014, Ogino et al., 2003).

The robustness of the rabies virus pNT was demonstrated by the correlation of VNAb titres obtained between the rabies pNT and the gold standard FAVN test. Low inter-assay variation is also supported by the high degree of correlation of VNAb titres when the pNT was performed using two different cell lines. Rabies VNAb titres measured by our pNT and the HIV(luc) pNT were also very closely correlated, despite using different retroviral systems, reporters and cell lines. The discordant results between our rabies virus pNT and the FAVN test were mostly clustered around the test cut-off titre, which has previously been reported when other pseudotype assays have been compared to the FAVN test (Wright et al., 2008, Wright et al., 2010). The samples used in this study had previously undergone at least 3 freeze-thaw cycles, which may have accounted for the VNAb in these samples becoming undetectable, as previous studies have shown that multiple freeze-thaw cycles can decrease the detectable titre of antibodies in serum (Boadella and Gortázar, 2011, Brey et al., 1994).

Our pNT includes MLV(VSV) as a non-specific neutralisation control which has been utilised in other pNTs as a measure of non-specific neutralisation (Logan et al., 2016). The presence of non-specific neutralisation and toxicity has been observed in many assays and has been recognised as having a role in false positivity in the FAVN test (Shiraishi et al., 2014a, Thrusfield, 1986). The non-specific neutralisation of one of the serum samples tested in this study highlights the necessity of this control, as without this control the sample would have been incorrectly reported as positive for rabies VNAb.

A further benefit of the pNT is that the volume of serum required per test is lower than with the FAVN or RFFIT (Cliquet et al., 1998). In this study we were able to reduce the volume of serum initially required by half, making the volume

of serum required for our assay just 45 µl compared to the 300 µl required in the FAVN test (OIE, 2012b). The overall objective of this study was to produce an assay which could be used to measure rabies VNAb in animals, particularly wildlife, therefore producing assays that require small volumes of sera are highly desirable, as in wildlife sampling only small volumes of sera are often available.

Ideally results from the pNT would be reported in IU/ml, but this would require the international OIE reference standard to be included in each assay, unfortunately during this study we were unable to obtain any additional control serum. We therefore had to use a surrogate control in our assay; this serum was diluted to a titre of 40, the same titre the OIE reference serum achieved in the rabies virus pNT. On all occasions this control measured 40, hence we are confident that this cut-off is representative of adequate protection. Additionally, the Advisory Committee on Immunisation Practices suggest sera that completely neutralises rabies virus at a dilution of 1:5 should be considered positive for rabies-specific antibodies, therefore our cut-off titre, a dilution of 1:40, is sufficient. If the OIE reference serum were to become available, this reference serum would replace our local control serum.

Our cross-neutralisation experiment confirms previous evidence that VNAb to rabies virus cross-react with other phylogroup 1 lyssaviruses, but provide no protection against lyssaviruses belonging to phylogroup 2 and presumably the more recently isolated lyssaviruses belonging to a putative third phylogroup (Horton et al., 2010). The FAVN is generally used for measuring rabies VNAb in response to vaccination, but for serosurveillance it should be borne in mind that the FAVN or rabies virus pNT cannot distinguish between VNAb to lyssaviruses of the same phylogroup. This may not be discriminatory enough for screening wildlife species, especially bats, from areas where multiple lyssaviruses are circulating. In these circumstances a positive VNAb result should be further investigated to determine the definitive source of the VNAb response. As reported here and in other studies (Wright et al., 2008, Wright et al., 2010) the highest VNAb titres are demonstrated to the particular lyssavirus eliciting the initial immune response.

Vero and BHK-21 are commonly used cell lines for culture-adapted strains of rabies virus (Thoulouze et al., 1998), however rabies strains have been shown to

infect a variety of cell lines of various origins (Jackson and Wunner, 2007). Although BHK-21 cells were our first choice of cell line for the rabies virus pNT, they proved to be unsuitable as pseudotype titres were considerably lower in BHK-21 cells than in other cell lines. The poor transduction efficiency of MLV(lyssavirus) pseudotypes in BHK-21 cells has also been previously reported in other MLV-based pseudotype systems, including those bearing rabies, Ebola and feline leukaemia virus glycoproteins (Wright et al., 2008, Wool-Lewis and Bates, 1998, Hussain et al., 2011). Interestingly, in rabies virus pseudotyping this was reversed when the MLV core was replaced with an HIV core (Wright et al., 2008), suggesting that the poor infectivity may be the result of inadequate integration or expression of the MLV genome, or post-entry restriction of MLV, as opposed to a direct effect of viral entry. It has also been reported that the use of BHK-21 cells in the FAVN is not recommended for measuring VNAb response to rabies vaccination if the administered vaccine was cultured in BHK-21 cells. It has been demonstrated that serum samples from animals immunised with such vaccines may possess anti-BHK-21 specific antibodies (Shiraishi et al., 2014a) which may interfere with the assay and distort genuine rabies VNAb titres. Therefore the use of CRFK cells in the pNT is preferential, as the same cell line can be used for all serum samples.

The results of our cell line infectivity experiment demonstrated that in adherent cell lines the infectivity profile of MLV(rabies) pseudotypes is similar to that of pseudotypes bearing the glycoproteins of the other 6 lyssavirus species investigated in this study. Conversely, there is a difference in the ability of the lyssaviral pseudotypes to infect the suspension cell lines. Rabies virus pseudotypes barely infected any of the suspension cell lines tested, whereas some of the other lyssaviral pseudotypes were able to transduce some of these cell lines. Both MLV(LBV) and MLV(DUVV) pseudotypes infect MT-4 and SUP-T1 cells to a significant titre, with MLV(DUVV) pseudotypes also infecting BMOX40 and 3201 cells. These results support the evidence of differential tropism patterns of the lyssaviruses (Koraka et al., 2012, Weir et al., 2013). There have been various cellular receptors proposed for rabies virus including nAChR, NCAM and p75NTR, but none have been demonstrated as essential for cell entry in all lyssavirus species (Lafon, 2005). The low-affinity nerve-growth factor receptor p75NTR has been shown to be an *in vitro* receptor for both rabies virus and

EBLV-2 (Tuffereau et al., 1998), but not for the other 5 lyssavirus species tested here. Our results also show a similar cellular tropism of rabies virus and EBLV-2, which both infect MT-4 cells at a very low level and do not significantly infect any of the other suspension cell lines. It is evident from this study that MLV(DUVV) pseudotypes are capable of entering all 8 cell lines investigated, previous studies have also revealed different tropism patterns of rabies and DUVV (Koraka et al., 2012). The cellular receptors of DUVV have not been determined definitively, but considering the natural route of infection of DUVV is via bat scratches as opposed to direct viral inoculation through a rabid animal bite, it is reasonable to consider there may be different viral tropisms between these two viruses. Unfortunately we do not have the capacity to test the tropism of live lyssaviruses to see if the infectivity patterns mimic that seen in pseudotype infection, although this would be of great interest. Considering the suspension cell lines tested were generally non-permissive to MLV(rabies) pseudotypes, an automated high-throughput method of screening for rabies VNAb using suspension cells and flow cytometry was not appropriate. A similar automated approach was considered using adherent cell lines, but in this case cells are required to be removed from the well and resuspended, which often results in clumping of the cells and an inability to test via flow cytometry, therefore we concluded the post-assay processing proved prohibitive for the pNT. Titres of MLV(VSV) pseudotypes in suspension cell lines were lower than that seen in the adherent cell lines (with the exception of BHK-21 cells) but were still considerable, therefore although they were poorly transduced by the MLV(lyssavirus) pseudotypes we were confident that the cells themselves were adequate.

Rabies is classified as a neglected disease which is responsible for thousands of human deaths per year. Improved serosurveillance is required to further assess the circulation and reservoirs of rabies virus, and serological surveys are also important in determining the effectiveness of different rabies vaccination campaigns and delivery strategies. The pNT developed in this study provides a sensitive and cost-effective method of measuring VNAb to lyssaviruses, which can be used in BSL-2. The majority of rabies infections occur in developing countries, where the high containment facilities required for handling live rabies virus are unavailable, this assay provides the capacity for these countries to

improve rabies virus surveillance in a cost-effective manner. The assay is also flexible and can be readily adapted for the detection of VNAb to novel lyssaviruses that are ever emerging.

Chapter 4. Practical applications of lyssavirus pseudotype neutralisation tests

4.1 Introduction

Testing for rabies virus antibodies is routinely used to confirm an immune response in both humans and animals following rabies vaccination. As international human and animal health agencies now work towards the global elimination of canine rabies (WHO, 2013), with mass dog vaccination a key element of the strategy (One Health, 2016), serological testing will continue to be important to ensure the immunogenicity and effective delivery of rabies vaccines in the field. Preliminary data suggest that village dogs in Africa respond well to commercial vaccines delivered under field conditions (Morters et al., 2014a), however uncertainty still remains regarding the immunogenicity of rabies vaccines delivered in a wide range of settings throughout Africa and Asia. Readily accessible serological assays are required to provide critical information about immune response and the effectiveness of vaccine delivery strategies in achieving the 70% vaccination coverage required to control canine rabies (WHO, 2004). However, such serological investigations in vaccinated dogs are seldom performed due to the lack of veterinary diagnostic facilities and the high cost of rabies virus neutralisation tests (VNTs).

There is a widespread perception that in an infected host rabies antibody develops only shortly before, or at the onset, of clinical symptoms, at which stage the disease is invariably fatal. For this reason serosurveys are rarely used to investigate the epidemiology of rabies virus, whilst they are commonly used to investigate infection patterns for other pathogens. However, there have been studies that suggest non-fatal rabies exposures occur in both humans and

animals (Table 1.2). Serosurveillance in unvaccinated populations, particularly wildlife, may have a role in rabies elimination programmes to provide supportive evidence to declare a region rabies-free. Although active surveillance and contact-tracing studies may be sufficiently powerful to detect rabies cases in dogs and other domestic species, over the two-year period required to declare an area free of rabies (Townsend et al., 2013), there remains concern that cases in wildlife could remain undetected.

In this Chapter we demonstrate the utility of the rabies virus pseudotype neutralisation test (pNT), optimised in Chapter 3, to detect rabies virus neutralising antibody (VNAb) in three distinct studies. In the first study a wide variety of species from the Russian Far East were screened for rabies VNAb as part of a disease management strategy. The second study comprises a serosurvey of 71 lion samples collected from the Serengeti National Park (SNP) over a nine year period. In the third study the longitudinal serological response to rabies vaccination was investigated in a cohort of slum dogs in Kenya.

4.2 Results

4.2.1 Rabies virus neutralising antibody screening in the Russian Far East

After validation of the rabies virus pNT we were approached by Wildlife Vets International (WVI), a British charity involved in wildlife conservation, who asked us to test a panel of wildlife sera for the presence of antibodies to rabies virus. This serology would form part of the disease surveillance strategy of the Amur leopard reintroduction programme in the Russian Far East.

The Amur leopard (*Panthera pardus orientalis*) is on the critically endangered list (Jackson and Nowell, 2008) and is considered the world's rarest wild cat. There are fewer than 70 animals remaining in the wild, the majority of which are in the Russian Far East with a handful of Amur leopards in the adjacent areas of China (WWF, 2015). A reintroduction programme for the Amur leopard was formally approved in 2015 (ZSL, 2015) and aims to repopulate the historic range of the Amur leopard in the Primorsky Krai region of the Russian Far East using zoo stock (Kelly et al., 2013). Reintroducing captive bred animals is considered a last resort for wildlife conservation (Rahbek, 1993), but due to low numbers and

the substantial loss of the Amur leopard's habitat, it is considered the only remaining option to avoid extinction of this species (Uphyrkina and O'Brien, 2003). An important aspect of the reintroduction programme is the disease management strategy, which aims to identify diseases which may threaten the reintroduced and remaining leopards. This is achieved by identifying diseases present in wildlife and domestic species in the proposed reintroduction region. Of the 22 infectious diseases recognised as important to the disease management strategy, rabies was ranked third in order of importance, below feline immunodeficiency virus and feline leukaemia virus (Miquelle and Murzin, 2003). Dr. Lewis of WVI stated that finances for veterinary investigations will be limited and therefore it is essential that the money available is used to the greatest effect (Miquelle and Murzin, 2003). The cost of rabies VNAb testing using conventional methods such as the RFFIT, FAVN or MNT would be prohibitive for such disease management strategies, however the rabies virus pNT is a low cost alternative. Additionally, such conventional methods require larger volumes of sera than are required in the pNT, in situations such as these, where a single serum sample requires multiple testing for a range of diseases, assays utilising low serum volumes are preferable. As our rabies virus pNT is both inexpensive and uses only 45 µl of serum, we were able to test serum samples for rabies VNAb as part of the disease management strategy of the Amur leopard reintroduction programme.

Rabies is still a significant disease in Eurasia, with urban dog rabies affecting Asia and wildlife rabies the predominant form in Europe (Kuzmin et al., 2004). In Russia rabies is primarily maintained by wild canids, with different rabies strains maintained by different species in different areas. In the Russian Far East arctic rabies is the predominant strain which is maintained by the red fox, racoon dog and the wolf, with mustelids and other terrestrial mammals only occasional hosts (Kuzmin et al., 2004). Figure 4.1 illustrates the number of rabies cases in Russia from 1989-2010, in both domestic and wild animals, showing the dramatic increase in wildlife rabies since 1995 (Vedernikov et al., 1999). While these data represent the whole of the Russian Federation, rabies serosurveillance data for the Russian Far East is particularly limited, with the Primorsky Krai region reporting only 12 rabies cases between 1989-2007, with only a minority of these cases in wildlife (Lubchenko, 2008).

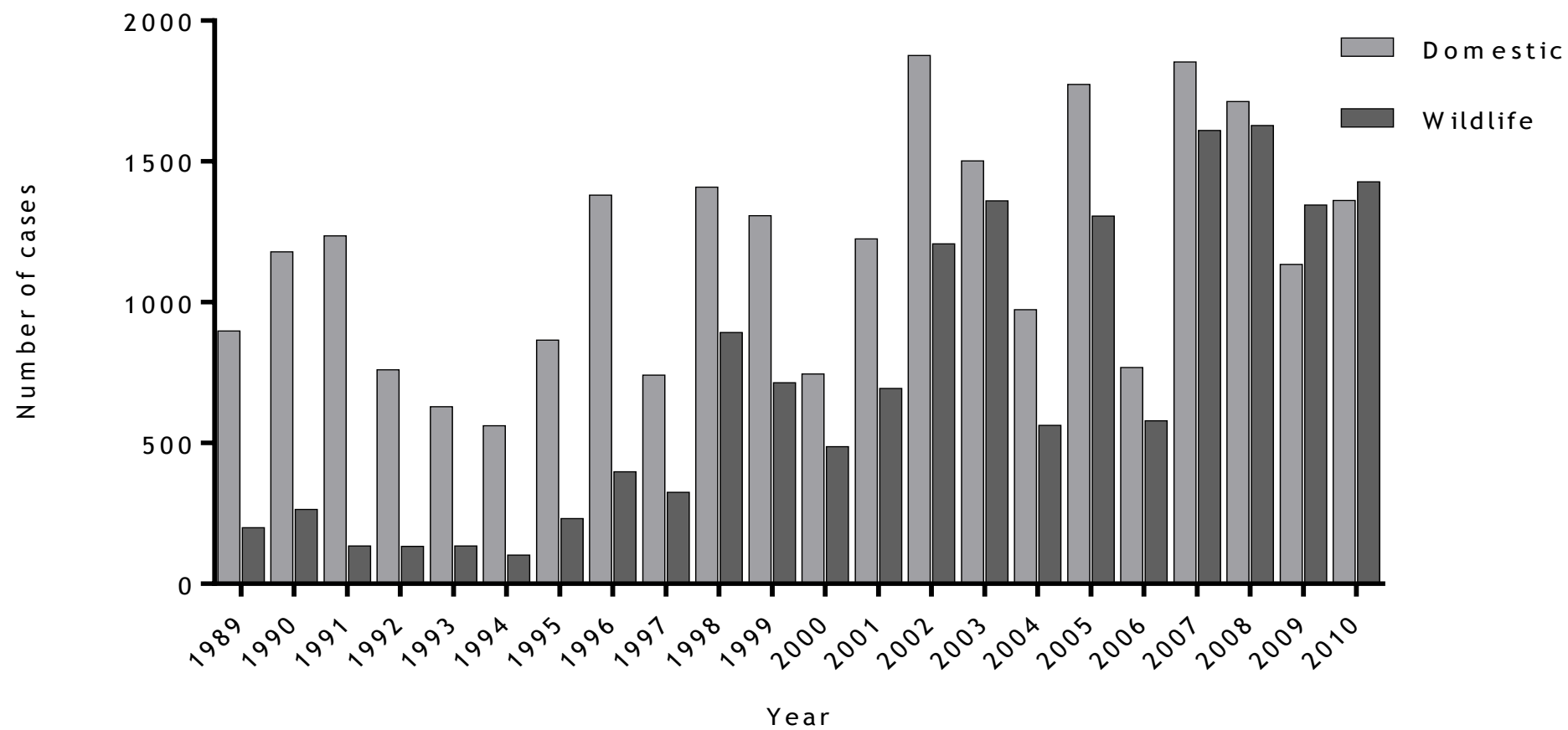


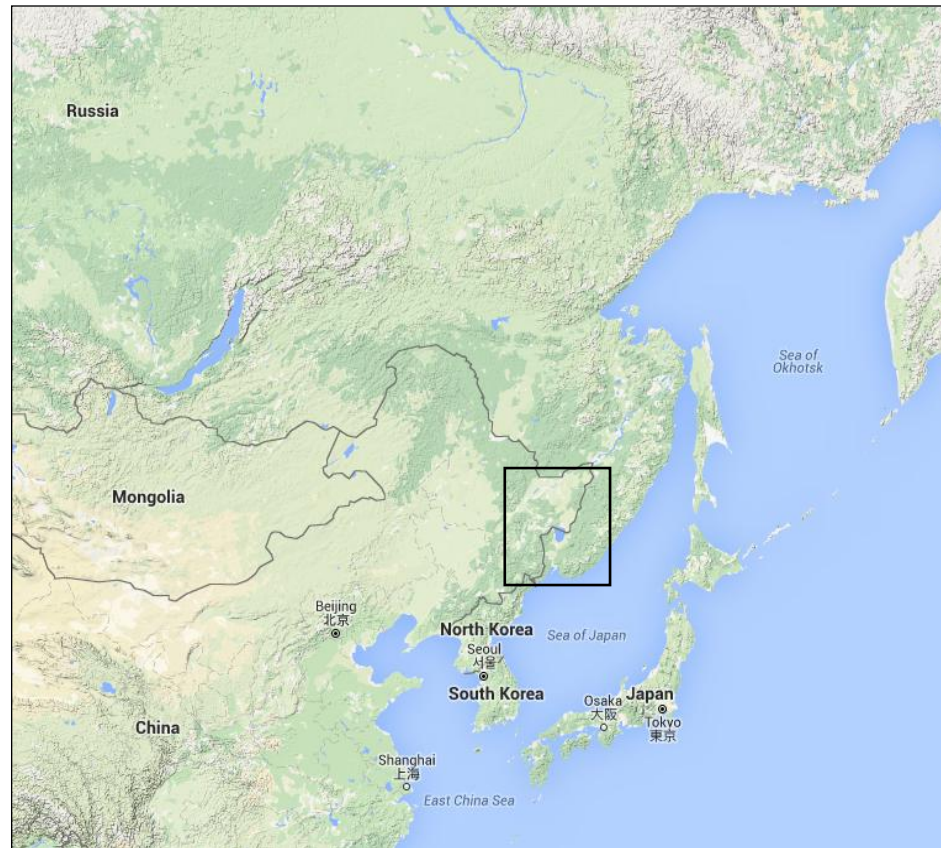
Figure 4.1 The number of rabies cases recorded in the Russian Federation from 1989-2010. The number of rabies cases in domestic and wild animals was collated from the Rabies Information System of the WHO Collaborating Centre for Rabies Surveillance and Research, representing the time period of collection of serum samples in this study (1989-2010).

To determine whether rabies was circulating in the area around the proposed release site of the Amur leopards (Figure 4.2), a total of 203 serum samples were tested for rabies VNAb by pNT. The panel of samples consisted of serum from 17 different domestic and wildlife species collected between 1989 and 2010 (Table 4.1), these samples had been collected and stored at -70°C for serosurveillance studies when funding became available. A cut-off threshold of 40 was chosen for the rabies virus pNT as evidence of rabies-specific VNAb in sera. One sample had a rabies VNAb titre of ≥ 640 , nine samples tested were inconclusive and the remaining 193 samples all had VNAb titres of ≤ 20 (Figure 4.3). The majority of these samples ($n=170$) did not neutralise rabies pseudotypes at a dilution of 1:10, the lowest dilution in the assay, and were therefore assigned a VNAb titre of <10 . Nineteen samples had a rabies VNAb titre of 10 and 4 samples measured 20 on the pNT. The sample with a high rabies VNAb titre was collected from a domestic dog sampled from a town in 2010. It is possible that this dog had previously been vaccinated as mass door-to-door vaccination campaigns are performed in that area (Lubchenko, 2008), however vaccination history from that dog was not available.

A positive VNAb titre in any of the wildlife serum samples would be a significant concern for WVI with respect to the Amur leopard reintroduction programme. To ensure that the neutralisation demonstrated in some samples at low serum dilutions (1:10 and 1:20) was not due to genuine rabies VNAb, two samples were additionally tested using the FAVN. As the FAVN is expensive, and funding for the disease management strategy of the Amur leopard reintroduction programme was so limited, only two samples were sent to Biobest, a private veterinary diagnostic laboratory, for testing on the FAVN. In this study four samples measured 20 by rabies virus pNT, these consisted of two samples from raccoon

dogs, one from a tiger and one from a vole. The decision of which samples test by FAVN was reached considering both the species from which the sample originated and the volume of serum available, as a minimum of 300 µl was required for FAVN testing. One raccoon dog sample was chosen for testing (#125), as raccoon dogs are the principal reservoirs for the arctic-like strain of rabies virus present in the Russian Far East (Kuzmin et al., 2004) and therefore the most likely species to have been exposed to the virus. The tiger sample (#09) was also selected for testing due to the implications for the Amur leopard if other wild felids in the area had been exposed to rabies virus. Both samples were negative when tested by FAVN, with a titre of 0.07 IU/ml.

a)



b)

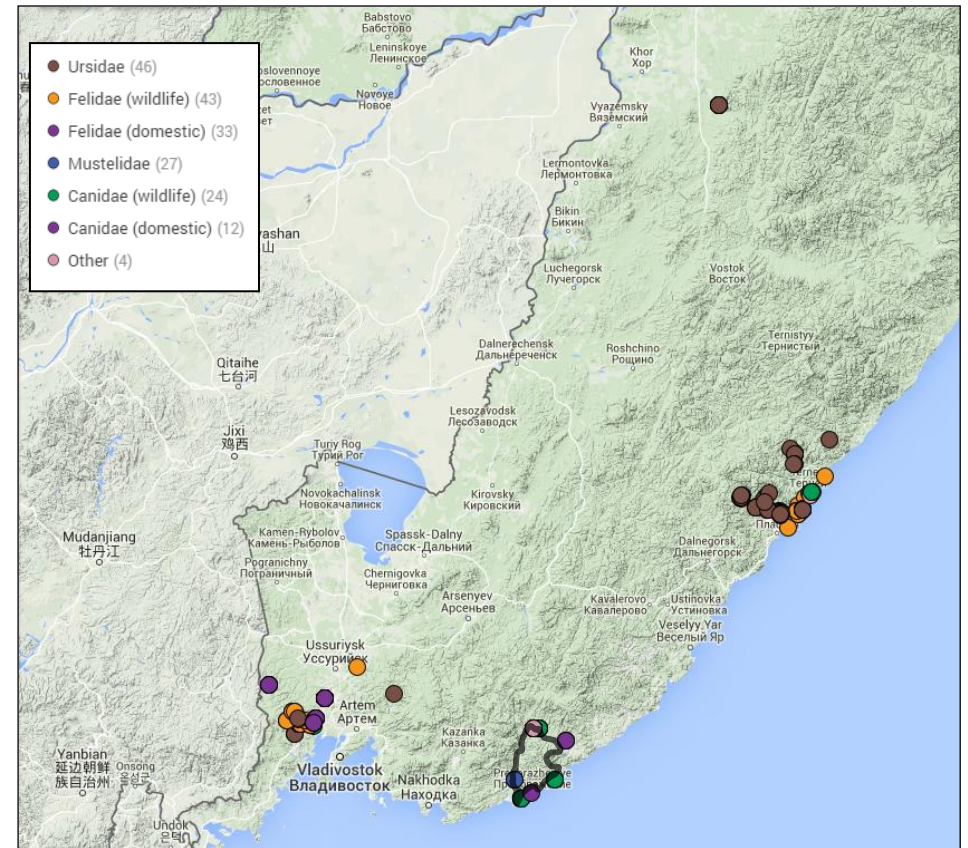


Figure 4.2 Locations of animals screened for rabies virus neutralising antibody. a) Map of the area of Russia where serum samples were collected and b) Map representing a zoomed in area of the square placed on Map a). Location points were plotted using latitude and longitude data provided with samples. The thick dark grey line represents the proposed area for reintroduction of the Amur leopard. Individual samples are represented by filled circles coloured by Family (n=189). Fourteen samples were not included as location data were not recorded for these samples. Maps were produced in Google My Maps (Map data ©2015 Google et al.).

Year of collection	American mink	Badger	Brown bear	Himalayan black bear	Cat	Feral cat	Dog	Feral dog	Eurasian lynx	Field mouse	Harbour seal	Hare	Leopard	Leopard cat	Raccoon dog	Red fox	Siberian weasel	Tiger	Vole	Total
1989			4	4																8
1990			5	1																6
1991				3																3
1993			1	1														1		3
1995			3																	3
1996				6														1		7
1997			1	6					2											9
1998			1	2					2									2		7
2000			1															2		3
2001									1						1			4		6
2002													2	1				6		9
2003		6		3									3	1	7		1	2		23
2004	1	17											2	2	14	1		2		39
2005		2	2	1														4		9
2006			1						1		1							1		4
2007																1		1		2
2008					1	3	1			1		1							1	8
2009					24		1	1												26
2010					15		10													25
Unknown					2		1													3
Total	1	25	19	27	42	3	13	1	6	1	1	1	7	4	22	2	1	26	1	203

Table 4.1 Species and year of collection of serum samples tested in this study. Serum samples from domestic animals and wildlife from the Russian Far East were submitted for testing for rabies VNAb, various numbers of samples were collected from 17 different species between 1989 and 2010 (n=203).

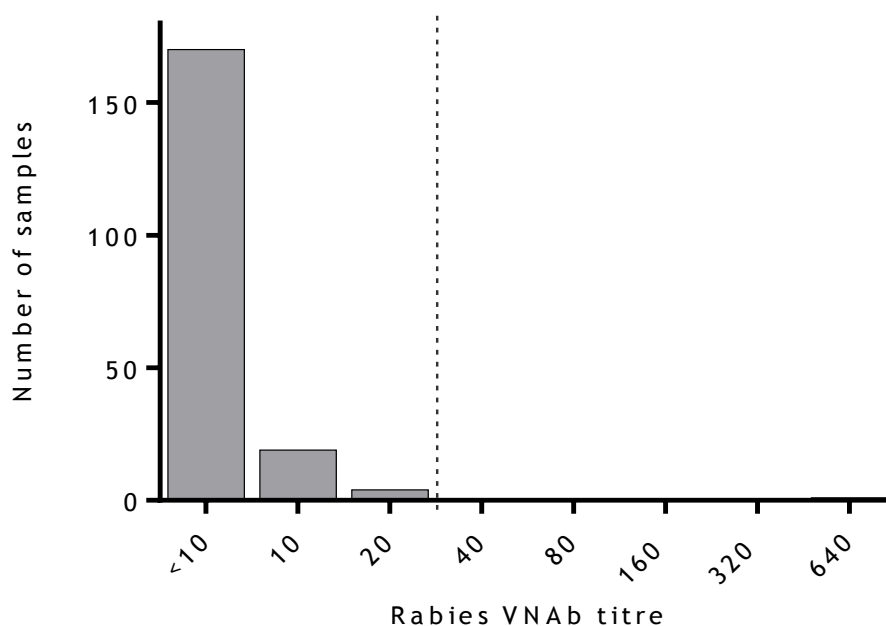


Figure 4.3 Rabies VNAb titres of serum samples tested in this study. Serum samples from domestic animals and wildlife from the Primorsky Krai region of the Russian Far East, collected between 1989 and 2010, were tested for rabies VNAb by the pNT (n=194). The vertical dashed line represents the cut-off titre of 40 utilised in this study.

Nine of the 203 samples (4%) tested for rabies VNAb were recorded as inconclusive, the causes of these inconclusive results included non-specific fluorescence, cytotoxicity or sample haemolysis of serum, with non-specific fluorescence causing the majority of inconclusive results in this study (Table 4.2). Non-specific fluorescence describes fluorescence observed in the test well which is not attributed to the GFP expressing pseudotype. Figure 4.4 illustrates

examples of non-specific fluorescence observed whilst testing the serum samples in this study. As demonstrated in the micrographs, fluorescent material present in the serum can entirely obscure the cell monolayer, therefore transduction of the target cells with MLV(rabies) pseudotypes cannot be observed (Figure 4.4 b). There may also be fluorescent components of serum which resemble GFP expression to an inexperienced user (Figure 4.4 c & d). The pNT could still be interpreted for some samples with non-specific fluorescence, for example the sample in Figure 4.4 d. Non-specific fluorescence can be removed by further diluting the serum, but this was not appropriate in this instance as we were interested in the neutralising activity of the serum at the lowest dilutions.

Sample number	Species	Date Collected	Reason for inconclusive result
10	Tiger	19/03/1998	Non-specific fluorescence
14	Tiger	10/10/2001	Non-specific fluorescence
32	Himalayan black bear	08/08/1989	Cytotoxicity
48	Brown Bear	28/05/2003	Non-specific fluorescence
69	Himalayan Black Bear	02/06/2003	Cytotoxicity
110	Raccoon dog	16/10/2003	Non-specific fluorescence
114	Raccoon dog	02/11/2003	Non-specific fluorescence
115	Raccoon dog	02/11/2003	Cytotoxicity
202	Hare	23/12/2008	Sample haemolysed

Table 4.2 Serum samples which were inconclusive for rabies virus neutralising antibody. Species, date of collection and the reason for an inconclusive result for serum samples which were recorded as inconclusive for rabies VNAb by pNT.

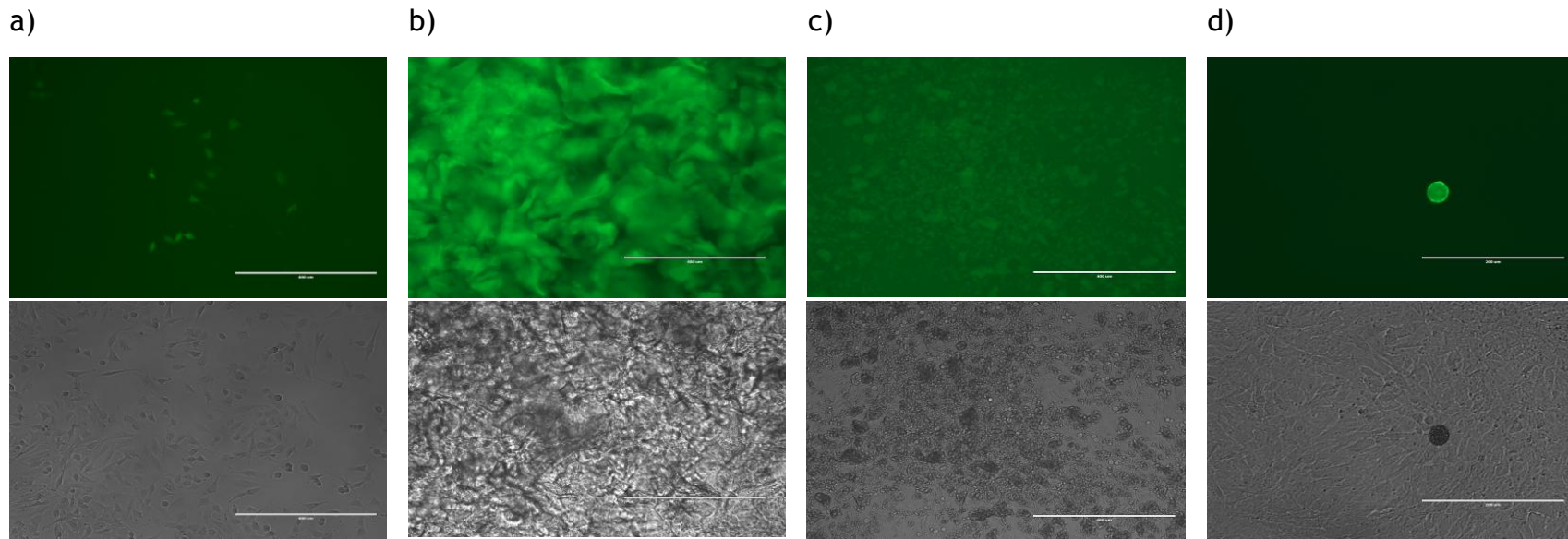


Figure 4.4 Fluorescence and light micrographs of the rabies virus pNT with test serum samples. Micrographs of wells of the rabies virus pNT for four serum samples (a-d) at the 1:10 dilution under fluorescence and inverted light microscope. a) CRFK cells infected with MLV(rabies) pseudotypes b-d) non-specific fluorescence in serum. Line represents scale bar (a, b, c: 400 μm , d: 200 μm).

4.2.2 Rabies virus neutralising antibody screening in African lions

In Tanzania rabies is endemic in the domestic dog population (Cleaveland et al., 2002) and there have been confirmed spillover infections in wildlife species within the SNP which have included a jackal, bat-eared fox, aardwolf, civet and leopards (Lembo et al., 2008). There have been few reported cases of rabies infection in lions globally (Berry, 1993, Pfukenyi et al., 2009), with reported cases from Namibia being directly attributed to hunting infected prey (Berry, 1993). East et al. (2001) have described rabies VNAb in spotted hyenas in the SNP with the absence of clinical disease. We hypothesise that due to similar ecosystems and prey species, lions may also show evidence of natural, non-fatal, rabies exposure.

Lion sera (n=71) collected between 1997 and 2005 from animals within the SNP were tested for rabies VNAb by pNT. The characteristics of the lions tested in this study are presented in Table 4.3. Rabies VNAb titres ≥ 40 were considered evidence of rabies exposure in this study. The overall seroprevalence of rabies VNAb in lions was 20% (95% CI 11-31%), with titres in rabies VNAb positive lions ranging from 40 to ≥ 640 , the highest recordable titre on the rabies virus pNT (Figure 4.5). Lion samples with VNAb were collected over many different years, with 21% of female lions and 12% of males seropositive.

Year	Age				Total
	1-3 y	3-5 y	5+ y	Unknown	
1997	-	1 (100)	5 (20)	3 (67)	9
1998	11 (64)	6 (50)	4 (50)	4 (33)*	25
1999	-	1 (0)	3 (0)	1 (0)	5
2000	-	1 (100)	-	1 (0)	2
2002	-	-	-	1 (0)	1
2003	-	5 (0)	5 (20)	4 (50)**	14
2004	7 (71)	2 (0)	1 (0)	3 (0)	13
2005	-	-	2 (0)	-	2
	18	16	20	17	71

Table 4.3 Year of sampling, sex and age data of lions tested for rabies VNAb in this study. Lion samples collected opportunistically from the Serengeti National Park between 1997 and 2005 were tested for rabies VNAb in this study. Lion populations have been monitored in the Serengeti since 1966 and most lions in this study have been observed since birth so exact age data were collected, lions without exact ages were classified as unknown. Numbers in brackets represent the percentage of male lions, values were calculated based on the total number of lions of known sex, *indicates that the sex of one or **two lions in the group were unknown.

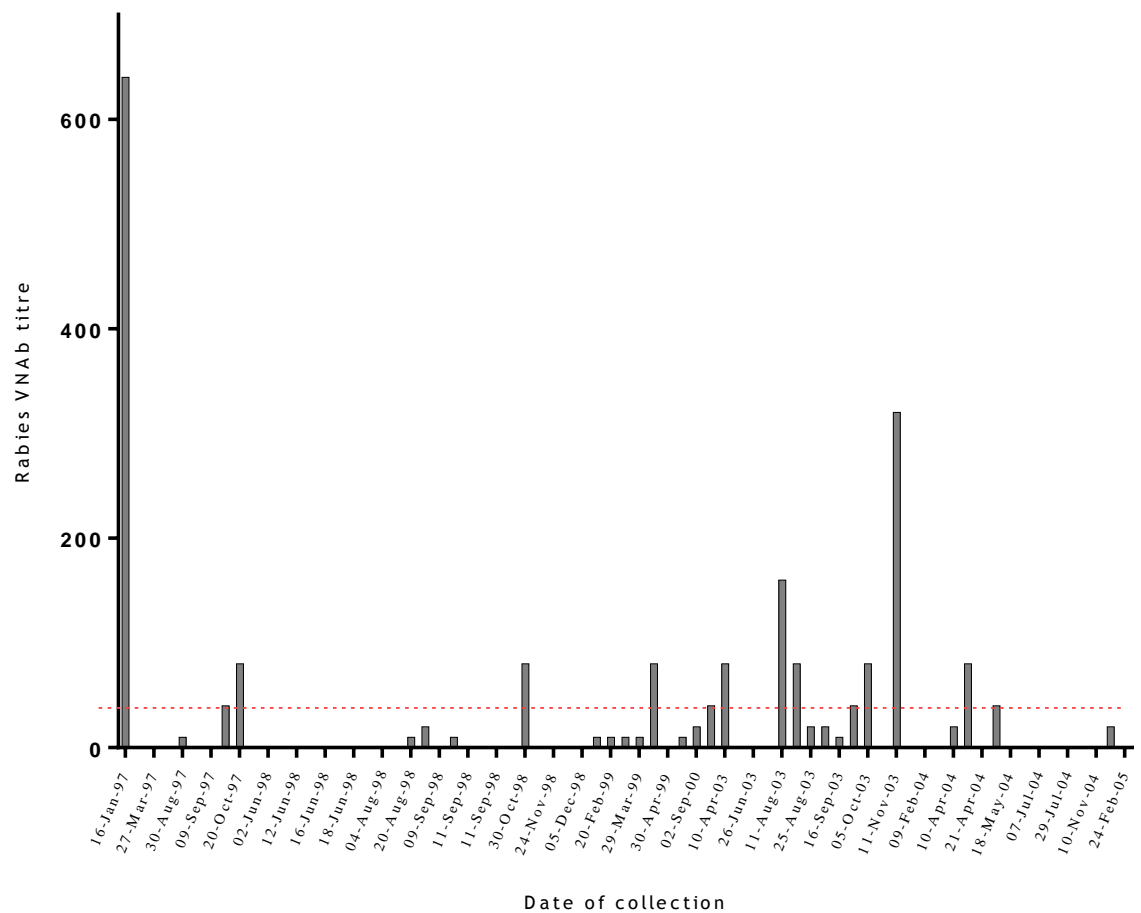


Figure 4.5 Rabies virus neutralising antibody titres of Serengeti lions. Lion samples collected between 1997 and 2005 were tested for rabies VNAB using the pNT. The red dashed line represents a titre of 40, the cut-off in this study for seropositivity to rabies virus. A subset of these samples was tested by Lynne Ferguson as part of a Masters project.

4.2.3 Investigating the virus neutralising antibody response to rabies vaccination in Kenyan slum dogs

Rabies vaccination of dogs in endemic areas is critical for controlling rabies, however the effectiveness of the vaccine in field conditions is poorly understood. Various individual level factors, such as malnutrition, may affect the serological response and duration of immunity (Morters et al., 2014a). In this study we utilise the rabies virus pNT to assess the VNAb response to rabies vaccination in a cohort of Kenyan slum dogs.

A large study, conducted in 2007, in which 637 dogs were sampled over the course of approximately 14 months, was undertaken to assess the use of dogs in Kenya as sentinels of infectious diseases (Halliday, 2010). Dogs were recruited from the informal settlement of Kibera in Nairobi, Kenya (Figure 4.6). Dogs in the study were classified as owned but were free-roaming, which is common place in slums such as Kibera (Morters et al., 2014b, Makau et al., 2015). As an incentive to dog owners to participate in the study, dogs were vaccinated against rabies virus at enrolment. This vaccination did not form part of the original study but with the development of the rabies virus pNT we were able to use these stored serum samples to explore the response to rabies vaccination in these dogs over the 14-month time period.

A total of 7 visits were made during the study period and attempts to collect serum samples from all recruited dogs were made at each visit. For this study, dogs were included if at least six serum samples had been collected over the course of the original study (n=67). The longitudinal nature of the original study not only allowed us to assess the immunological response to rabies vaccination but also to analyse the kinetics of rabies VNAb over time. In addition to serum samples being collected at each visit, in depth epidemiological data was also recorded at each time point.



Figure 4.6 Map of Kenya illustrating the location of the Kibera study site.

The Kibera study site was sub divided into 10 clusters (Figure 4.7) and each dog in the study was assigned to a cluster based on their household location. A total of 67 dogs were included in this study each having 5 ($n=1$), 6 ($n=36$) or 7 ($n=30$) serum samples collected during the original study (one sample was missing from dog #160 therefore only 5 samples were available for this dog). Serum samples and epidemiological information was collected at each time point (tp), data collected from dogs included dog identifier, age, sex, cluster, body condition, whether dogs were from a single or multi-dog household and the date of sample collection. Age, sex and cluster of the dogs included in this study are described in Table 4.4. Males and females were fairly evenly represented in the study cohort with 54% of dogs being male. The majority of the dogs in the study were adults, with only 15 (22%) dogs aged less than one year at enrolment. Age was reported by the owner at enrolment and ages were extrapolated from this point throughout the study. Dogs were grouped into age categories of 3-6 m, 6-12 m, 1-4 y, 4+ y, or Adult where no specific age was given but the dog was not juvenile. Body condition was measured on a scale of 1-5 where 1 is thin, 3 is ideal and 5 is obese (GFAH, 2007).

At initial enrolment (tp 0) serum samples were collected immediately prior to rabies vaccination being administered to all dogs in the study; further samples were then collected at tp 1-6. Serum samples collected at tp 0-4 were spaced at approximately 28 day intervals, there was then a gap of approximately 9 months, after which samples at tp 5 and 6 were collected, again with approximately 28 days between sampling. Households were visited in a similar order at each time point therefore the number of days between sampling was similar for each dog. Not all dogs were available for sampling at every visit, a total of 431 samples were included in this study (Table 4.5). Each sample collected was tested for rabies VNAb by pNT. Samples from the same dog were tested on the same assay run to reduce inter-assay variation between VNAb measurements from the same dog. Samples were initially tested at serum dilutions of 1:10 to 1:640. Samples with a VNAb titre of ≥ 640 , the highest measurable titre on the assay, were repeat tested with increasing serum dilutions up to 1:40960, therefore the highest recordable VNAb titre in this cohort was ≥ 40960 . Samples that were negative at the first dilution of 1:10 were recorded as having a rabies VNAb titre of <10 . For statistical analysis these samples were assigned an arbitrary rabies VNAb titre of 1.



Figure 4.7 Map demonstrating Kibera study site clusters. The Kibera study site was divided into 10 clusters based on existing geographical boundaries. Each dog in the study was assigned to a cluster based on the location of the owner's household. Figure adapted from the original map created by the Kenya Bureau of Statistics.

	Age					Total	
Cluster	3-6 m	6-12 m	1-4 y	4+ y	Adult		
	1	-	-	-	4 (50)	-	4 (50)
	2	4 (75)	2 (100)	3 (67)	4 (75)	6 (50)	19 (68)
	3	1 (0)	-	-	1 (100)	-	2 (50)
	4	2 (50)	-	1 (100)	1 (100)	3 (0)	7 (43)
	5	1 (100)	1 (100)	-	3 (67)	3 (33)	8 (63)
	6	1 (0)	3 (67)	2 (50)	1 (100)	6 (17)	13 (38)
	7	-	-	-	1 (100)	1 (0)	2 (50)
	8	-	-	1 (0)	2 (100)	1 (0)	4 (50)
	9	-	-	1 (0)	-	2 (50)	3 (33)
10	-	-	2 (0)	1 (100)	2 (100)	5 (60)	
Total	9 (56)	6 (83)	10 (40)	18 (78)	24 (33)	67 (54)	

Table 4.4 Characteristics of dogs enrolled in this study. Distribution of dogs in the study based on cluster, sex and age at enrolment. Numbers in brackets represent the percentage of male dogs.

Time point	Days post vaccine	Number of dogs sampled	Median titre	Titre range
0	0	67	40	<10-320
1	28-29	65	2560	40- \geq 40960
2	56-57	65	320	20-20480
3	84-90	63	160	20-2560
4	113-118	49	160	10-2560
5	369-403	62	80	<10-320
6	397-438	60	160	10- \geq 40960
Total number of samples		431		

Table 4.5 Distribution of sample data for each time point in the study.

Serum samples were collected from dogs at enrolment prior to administration of rabies vaccine and then at a further 6 time points post vaccination. Not all dogs were available for sampling at every time point.

4.2.3.1 Pre-vaccination rabies virus neutralising antibody titres in dogs

As a baseline value rabies VNAb titres were measured in dogs at tp 0, collected immediately prior to vaccination. Thirty four (51%) of the dogs in this study had a pre-vaccination titre of ≥ 40 , the VNAb titre in the rabies virus pNT which corresponds to ≥ 0.5 IU/ml (the titre considered protective). Two dogs had titres as high as 320 (Figure 4.8). Rabies VNAb titres in unvaccinated slum dogs was unexpected as all dogs included in this study were recorded as having never received a rabies vaccination, and rabies vaccination campaigns had not been conducted in the area prior to the study. To consider whether this finding was an artefact of the pNT, we tested 30 serum samples from UK dogs as a comparison. Of these samples, 29 had rabies VNAb titres of <10, the lowest recordable titre on the rabies virus pNT and one sample had a rabies VNAb titre of ≥ 640 . It is

likely that the sample with the very high rabies VNAb titre is from a dog vaccinated against rabies, although as the samples were anonymised prior to testing we were unable to confirm this. The lack of rabies VNAb titres in the UK dogs increased our confidence that the rabies VNAb titres observed in the unvaccinated Kenyan dogs were genuine and not an artefact of the assay. As the MLV(VSV) control pseudotype was also included in our assay we could be confident that these rabies VNAb titres were not the result of non-specific neutralising activity.

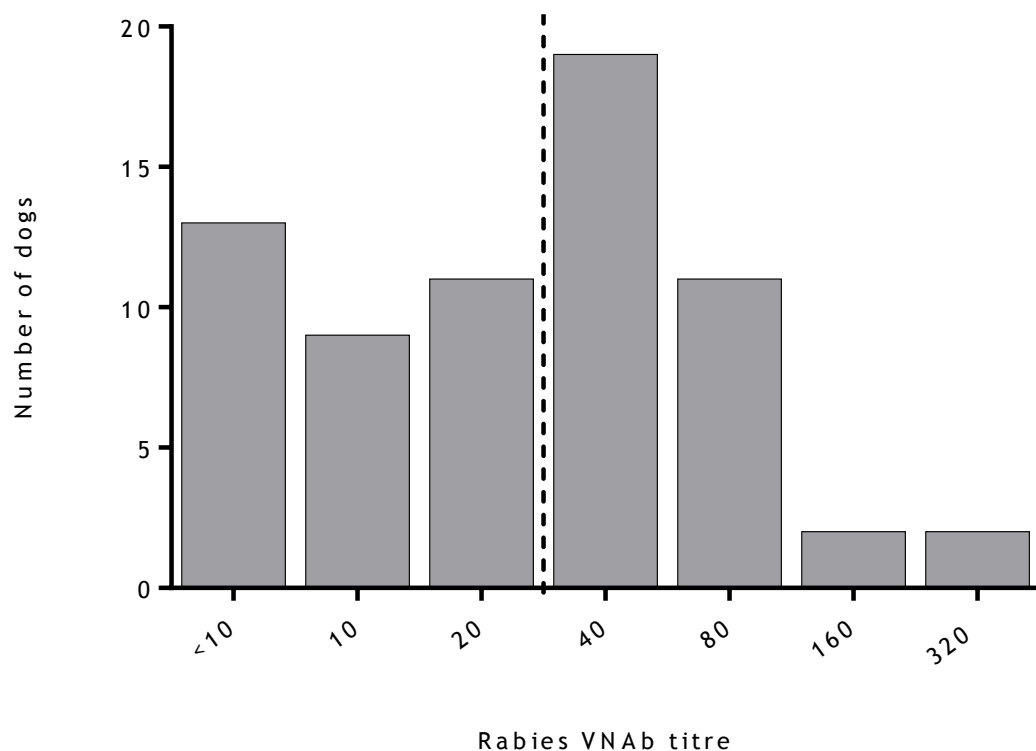


Figure 4.8 Rabies virus neutralising antibody titres in pre-vaccination samples. Rabies VNAb titres were measured in dog sera collected immediately prior to rabies vaccination (n=67). The dashed line represents the titre considered to be protective against rabies virus challenge (≥ 40).

The rabies VNAb titres detected in reportedly unvaccinated dogs from the Kibera study site may be the result of natural exposure to rabies virus in these dogs, or possibly the detection of cross-neutralising antibodies to other lyssaviruses or even other pathogens. We cannot rule out owners incorrectly reporting vaccination status of the dog, however, we consider it unlikely that many dogs in this community would have previously received rabies vaccination. Furthermore, these results are consistent with reports of rabies VNAb in previously unvaccinated dogs elsewhere in rabies endemic settings in Africa (Morters et al., 2014b, Cleaveland et al., 1999, McNabb, 2008).

4.2.3.2 Kinetics of rabies virus neutralising antibody in vaccinated dogs

Dogs were followed for over one year. The median rabies VNAb titres increased sharply between pre-vaccination (tp 0) and 28 d post vaccination sampling (tp 1) and then declined from tp 1 to tp 5 (Figure 4.9 and Figure 4.10).

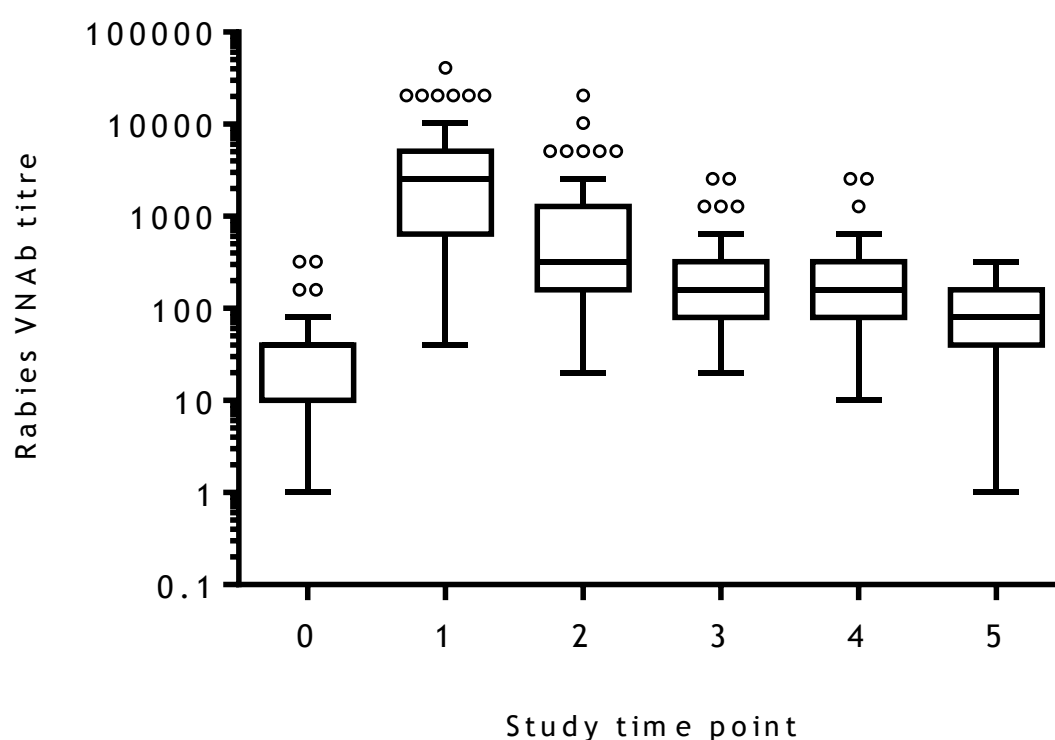


Figure 4.9 Variation in rabies virus neutralising antibody titre at each time point from pre-vaccination to approximately 1 year post vaccination. Tukey's boxplot demonstrating median, upper and lower quartiles of rabies VNAb titres for all dogs sampled at time points 0-5. Whiskers represent 1.5x interquartile range and circles represent outliers (n=371).

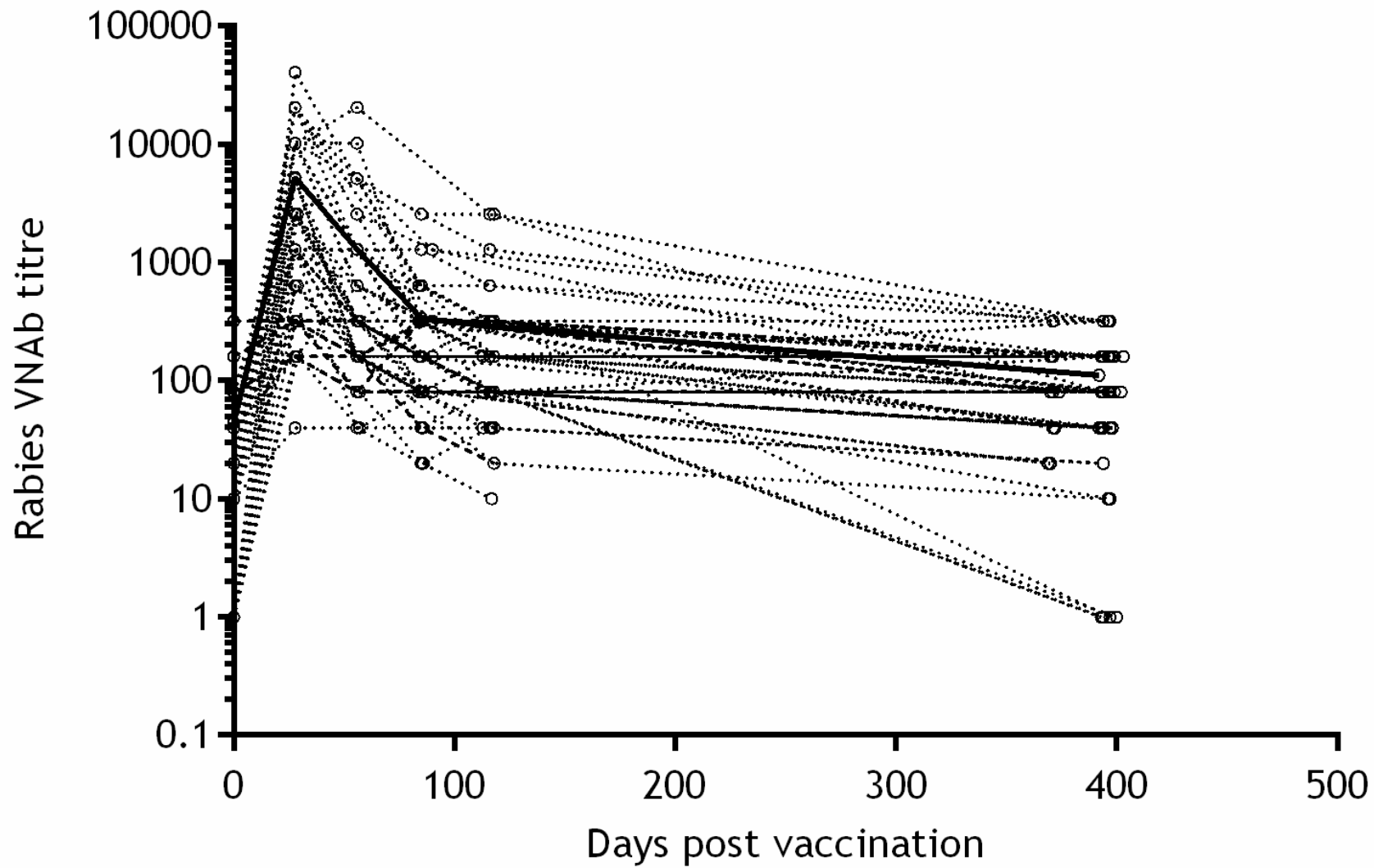


Figure 4.10 Rabies virus neutralising antibody titres for individual dogs from pre-vaccination to approximately 1 year post vaccination. Rabies VNAb titres of all dogs in the study for which a serum sample was collected at time point 1 (n=65). Individual dogs are represented by dashed lines and circles indicate day of sampling. The median titre is represented by the solid black line.

All dogs, except one, demonstrated an increase in rabies VNAb titre from the pre-vaccination titre to the 28 d post vaccination titre; the dog that did not exhibit an increase in titre had a titre of 320 at both tp 0 and tp 1. At 28 d post vaccination all dogs achieved a rabies VNAb titre of at least 40, which in this assay is equivalent to the 0.5 IU/ml considered protective against rabies virus challenge. However, there was significant variation in rabies VNAb titre between dogs at tp 1, with titres ranging from 40 to ≥ 40960 with a median titre of 2560 (Figure 4.9).

Variation in the magnitude of VNAb response to rabies vaccination in these dogs was investigated using general linear models. The difference in log titre between tp 0 and tp 1 VNAb titres was used as the response variable (hereafter referred to as magnitude of response). Log titre at pre-vaccination sampling (tp 0) was tested as a continuous explanatory variable. Sex, condition, and age (categorised into 3-6 m, 6-12 m and >12 m) at vaccination were considered as categorical explanatory variables. Interactions between pre-vaccination titre and each of the categorical variables were also tested. Interactions between categorical variables were not considered, as a high proportion of combinations of levels had either no or very few observations. All dogs with complete information for all variables were included in the model (n=61), three dogs were excluded from the model as there was no condition recorded at the time of vaccination and one dog was excluded as the magnitude of response was zero.

Under a backward elimination procedure, nested models were compared using likelihood ratio tests to determine which of the explanatory variables should be included in the final model (Table 4.6). Model selection indicated that pre-vaccination titre ($p < 10^{-10}$) and dog condition ($p < 0.005$) were significantly

correlated with the magnitude of response to rabies vaccination, this optimal model is represented in Figure 4.11. A negative relationship was observed between pre-vaccination titre and the magnitude of response to vaccination (Figure 4.12), with an increase in pre-vaccination titre of 1 log resulting in a reduced response to vaccination of 0.96 log on average (95% CI 0.72 - 1.19). The magnitude of response to vaccination for dogs of condition 2 or 3 was higher than of condition 1 dogs. After accounting for variation in response attributable to differences in pre-vaccination titre, condition 2 dogs exhibited a response to vaccination 2.14 log higher than condition 1 dogs (95% CI 0.87-3.42) and condition 3 dogs showed a response 1.56 higher than condition 1 dogs (95% CI 0.27-2.84). Confidence intervals for the coefficients for conditions 2 and 3 overlapped considerably indicating that dogs of these conditions did not react significantly differently to vaccination. The coefficient of determination for this model ($R^2=0.57$) indicated that pre-vaccination titre and condition at vaccination explained a considerable amount of the variation in the magnitude of VNAb response.

Model	AIC	Log likelihood	Degrees of freedom	p-value
$T0+S+C+A+(T0*S)+(\underline{T0*C})+(T0*A)$	231.2	-102.6	13	0.907
$T0+S+C+A+(\underline{T0*S})+(T0*A)$	227.5	-102.7	11	0.192
$T0+\underline{S}+C+A+(T0*A)$	227.5	-103.8	10	0.374
$T0+C+A+(\underline{T0*A})$	226.5	-104.2	9	0.600
$T0+C+\underline{A}$	223.6	-104.8	7	0.433
$T0+\underline{C}$	221.5	-105.7	5	0.005
$T0$	228.9	-111.4	3	1.53^{-10}
Intercept model	269.6	-132.8	2	-

Table 4.6 Values associated with model selection used to derive the model of magnitude of response to rabies vaccination. Model formulae demonstrate variables included in the model ($T0$ =pre-vaccination titre, S =sex, C =condition, and A =age, interaction variables take the form $(T0*S)$). For each model, AIC, log likelihood and degrees of freedom used in the model are shown. p-values are shown for the emboldened and underlined term in each model. These were derived from likelihood ratio tests between the model shown inline and the nested model below.

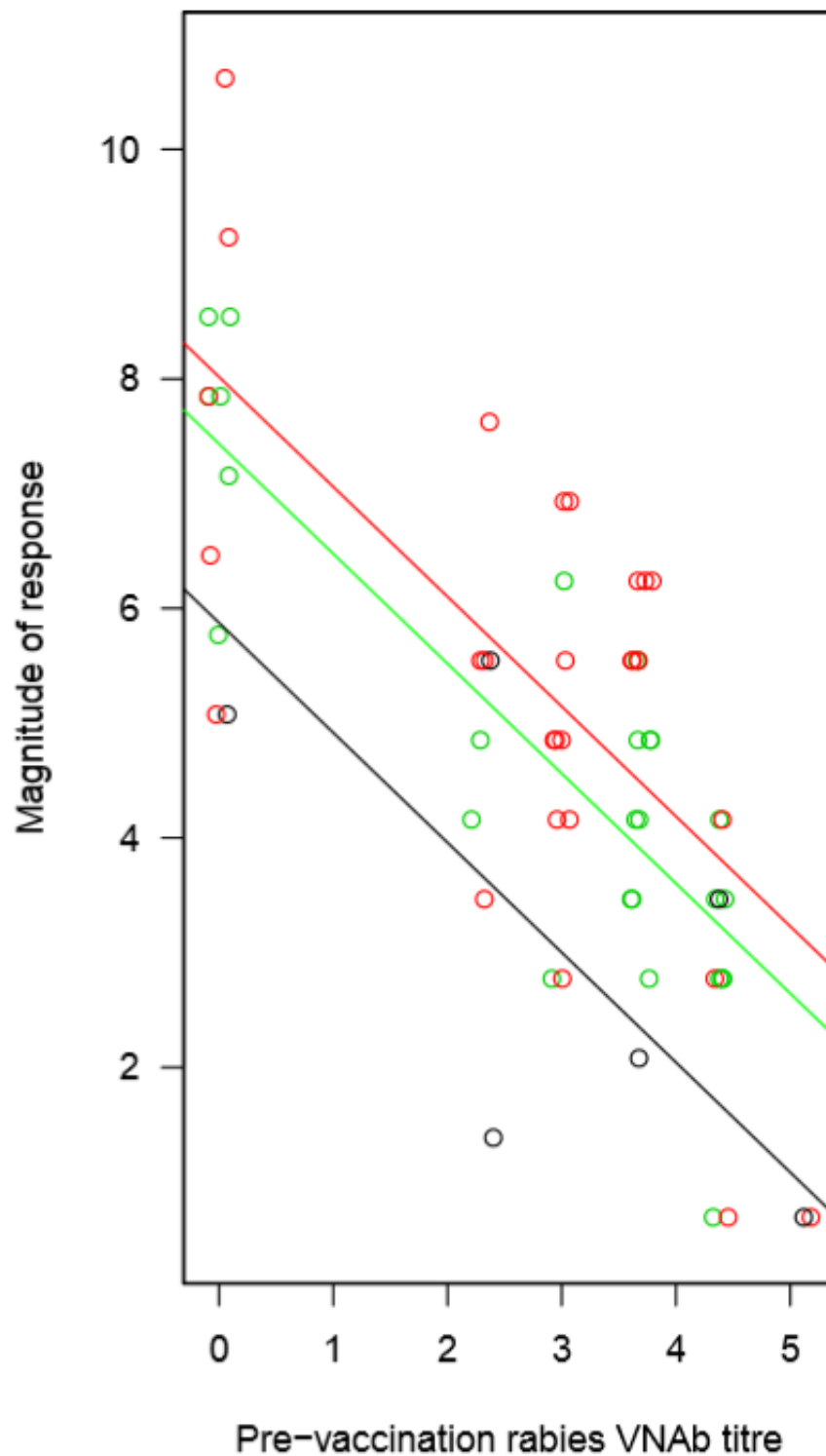


Figure 4.11 Variation in magnitude of response to vaccination. Increase in rabies VNAb titre plotted against pre-vaccination titre. A small amount of random displacement in the x-axis has been added to the position of points to allow visualisation of overlapping data points. Dog condition at vaccination is indicated by colour (1=black, 2=red, 3=green). Lines indicate the fitted values from a general linear model with parameters for pre-vaccination titre and condition at vaccination.

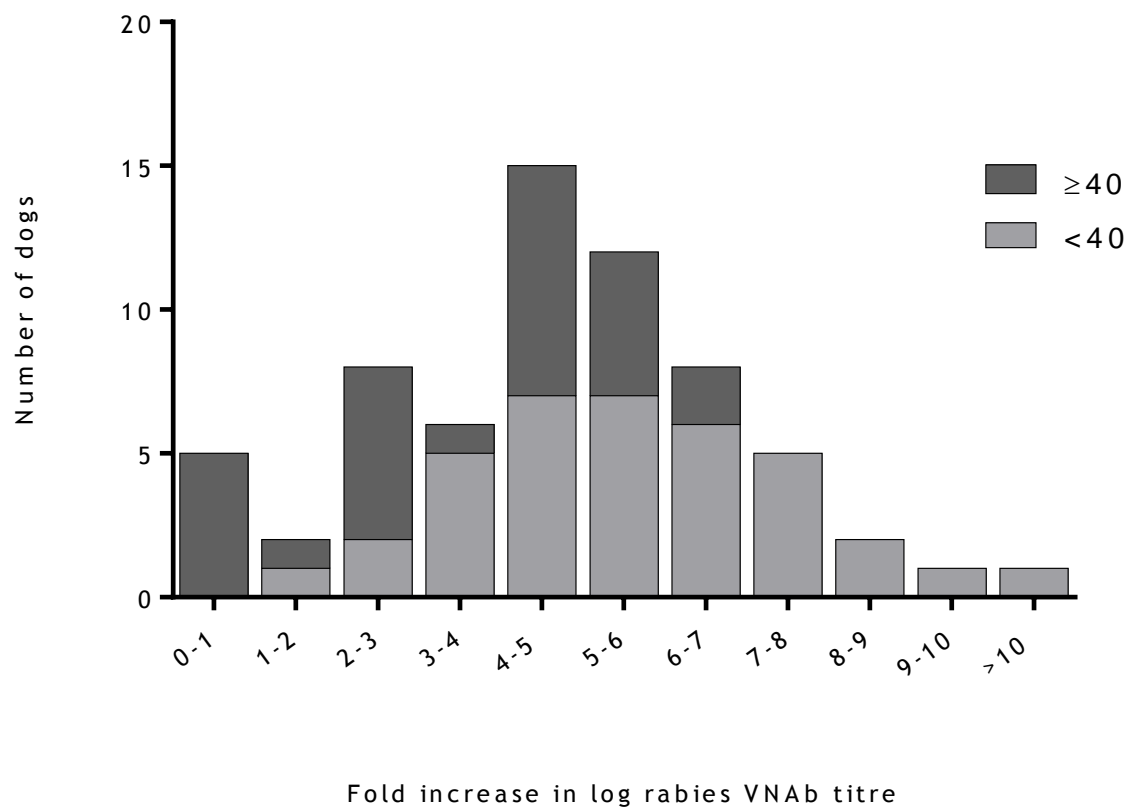


Figure 4.12 Magnitude of rabies virus neutralising antibody response at 28 days post vaccination in relation to pre-vaccination virus neutralising antibody titre. The magnitude of vaccine response was calculated using the fold increase in the log value of the VNAb titre at time point 1 compared to the log value of the pre-vaccination titre (tp 0) (n=65). Bars are coloured relating to their pre-vaccination VNAb titre.

The range of titres at tp 5 (approximately 1 year post vaccination) resembled pre-vaccination titres (Figure 4.9), ranging from <10-320 with a median titre of 80 (Figure 4.13). Fifty two dogs (84%) in the study had a rabies VNAb titre of ≥ 40 at tp 5, equivalent to a titre considered adequate protection against rabies challenge. However, using this cut-off ten dogs would be considered to have an inadequate rabies VNAb titre at 1 year post vaccination.

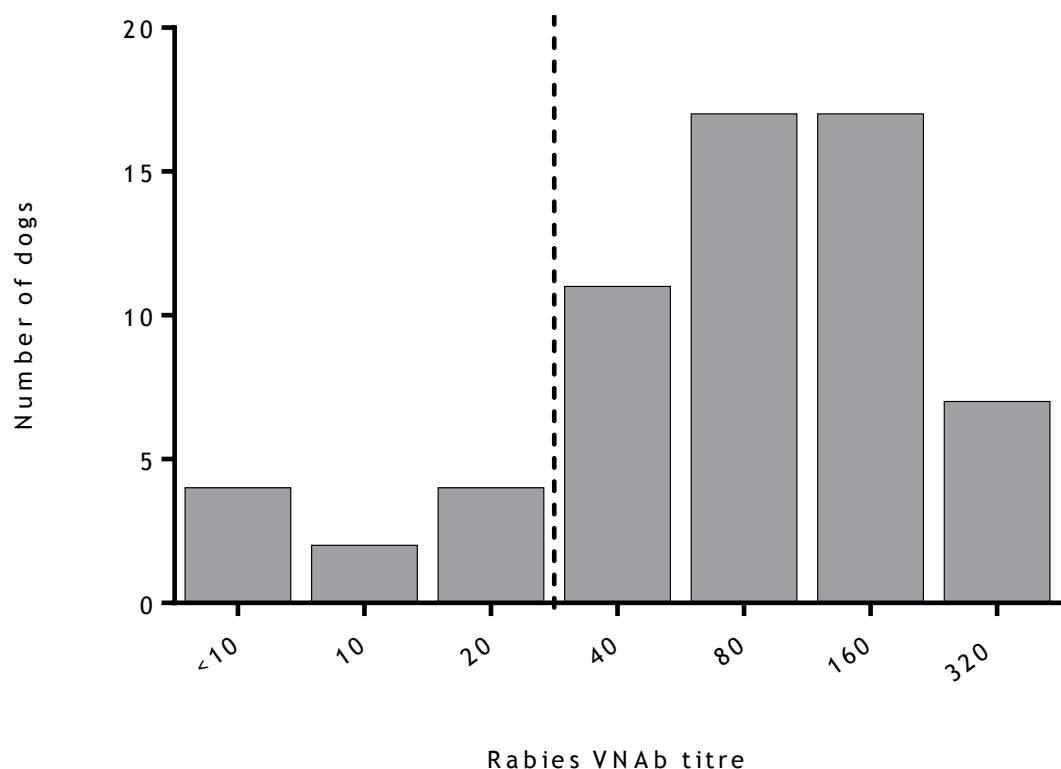


Figure 4.13 Rabies virus neutralising antibody titres for dogs in the study at approximately one year post vaccination. Rabies VNAb titres for dogs in the study, for which a sample was collected at time point 5 (n=62), which ranged from 369-403 days post vaccination. The dashed line represents the titre considered to be protective against rabies virus challenge (≥ 40).

A generalised linear model was used to test a variety of biologically plausible explanatory variables as predictors of a protective antibody response at 1 year post vaccination. Dogs were classified as protected (VNAb titre ≥ 40) or not protected (VNAb titre < 40) at tp 5 and this binary value was modelled as the response variable. Log titre at 28 d post vaccination (tp 1) was tested as a continuous explanatory variable. Sex, condition, and age (categorised into 3-6 m, 6-12 m and > 12 m) at vaccination were considered as categorical explanatory variables. Once again interactions between categorical variables were not tested due to high proportions of level combinations with zero or very few observations. Given that the interaction term between tp 1 titre and categorical variables resulted in fitted probabilities of 0 or 1, these interaction terms were not included in the model. All dogs with complete information for all variables were included in the model ($n=57$). Nested models were compared using likelihood ratio tests to determine which of the explanatory variables should be included in the final model. There were no statistically significant variables in this model that predicted protection (VNAb titre ≥ 40) at tp 5. The same explanatory variables were tested again using a general linear model with the binary response variable (protected/not protected) replaced by log titre at tp 5. In this model VNAb titre at tp 1 was statistically significant ($p < 0.05$). On average, an increase of 1 in log titre at tp 1 resulted in a 0.32 increase in log titre at tp 5 (95% CI 0.07-0.57), however the relationship between titre at tp 1 and tp 5 explained only a very small proportion of the observed variation in titre at tp 5 ($R^2=0.087$). The rabies VNAb titres in the 10 dogs in this study considered not protected at tp 5 are shown in Table 4.7.

Dog ID	Age	Sex	Condition	Time point 5 titre	Time point 0 titre	Time point 1 titre
29	Adult	F	2	<10	<10	ND
76	Adult	F	2	<10	20	320
103	3-6 m	M	1	<10	<10	160
112	4+ y	M	3	20	40	5120
114	4+ y	M	2	10	<10	10240
122	Adult	M	3	<10	<10	5120
140	4+ y	F	3	10	40	5120
185	Adult	M	3	20	10	640
207	Adult	F	2	20	20	1280
217	Adult	M	2	20	20	2560

Table 4.7 Characteristics of dogs with rabies virus neutralising antibody titres of <40 at approximately 1 year post vaccination. Data regarding age, condition, pre-vaccination and 28 d rabies VNAb titre for dogs which had a rabies VNAb titre of <40 (n=10) at time point 5 (ranging from 393-400 days post vaccination in these dogs). Age and condition data represents classification of the dog at the time of vaccination. ND=not done.

4.2.3.3 Repeatability of the pseudotype neutralisation test and use of the VSV control in field samples

In Chapter 3 we tested a small number of serum samples (n=12) in duplicate to assess the robustness of the rabies virus pNT. To improve our confidence in the reproducibility of the assay, in this study we retested a further 47 serum samples with varying titres ranging from <10 to 10240. Samples were tested on a different day using a new batch of MLV(rabies) pseudotype. Twenty-three samples had identical rabies VNAb titres on repeat testing, 23 samples had a titre that varied by one dilution and one sample was three dilutions higher on repeat testing (Figure 4.14). A high degree of correlation of VNAb titres was observed between the assays performed on different days (n=47, $R^2=0.83$, $p<0.0001$). Utilising a cut-off of 40 to classify titres as protective or not protective, three of the 47 samples had discordant test results between assays. These three samples would have been classified as negative for rabies VNAb on the first test but positive on the second assay. These samples all measured 40 on secondary testing and were therefore at the lowest titre considered positive.

In the rabies virus pNT each serum sample is additionally tested against VSV. If the rabies VNAb titre is >20 and the VSV titre is ≥ 40 , the sample is repeat tested against MLV(VSV) pseudotypes at higher serum dilutions, in order to detect non-specific neutralisation which is a documented cause of false positivity in the VNT (Mani and Madhusudana, 2013). In this study it was necessary to perform further MLV(VSV) pseudotype testing on four (0.9%) of the Kenyan dog samples (#121, #231, #256 tp 2 and #256 tp 3). Three samples had VSV titres of 40 on repeat testing (#121, #231, #256 tp 2) with rabies VNAb titres of 160, 320 and 20480 respectively. Sample #256 tp 3 had a VSV VNAb titre of 80 (rabies VNAb titre 2560). In each case the rabies VNAb titre was at least four-fold higher than VSV, therefore we can deduce that the rabies titres recorded are genuine, as any non-specificity had been diluted out.

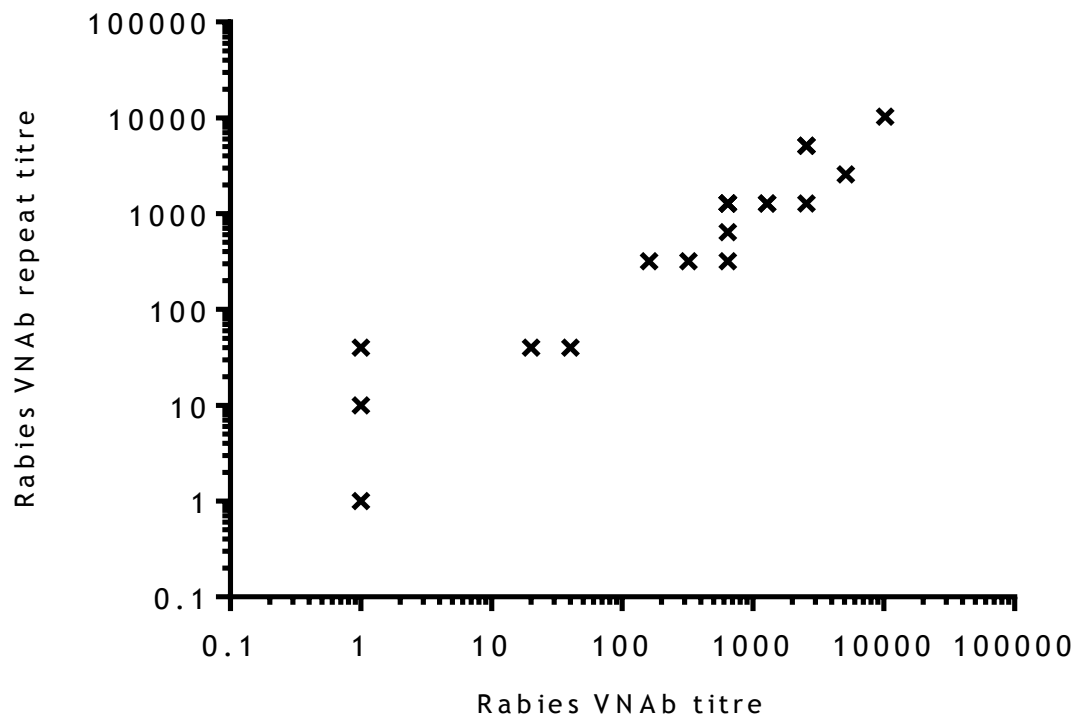


Figure 4.14 Repeatability of the rabies virus pseudotype neutralisation test. A selection of serum samples were tested twice for VNAb by the rabies virus pNT to determine the repeatability of the assay ($n=47$, $R^2=0.83$, $p<0.0001$). Coefficient of determination and p-values were calculated using Pearson's product-moment correlation.

4.2.3.4 Evidence of natural exposure to rabies virus over the course of the study

Final serum samples were collected in the study at tp 6, prior to a booster rabies vaccination being administered to a subset of dogs in the study group. These samples were collected between 397-438 days post initial vaccination. At this time point, 25% of dogs (n=15) had a four-fold or greater increase in rabies VNAb titre compared to tp 5 titres (Figure 4.15), suggesting a re-exposure to rabies antigen. In these dogs the fold increase in titre between tp 5 and 6 ranged from 4-fold to 10240-fold (Table 4.8). A four-fold increase in titre was not seen at any other time point in the study other than at initial seroconversion to rabies vaccination, with the exception of two dogs (#07 and #276) whose titres peaked at day 56 as opposed to day 28. There were no recorded rabies booster vaccinations administered to these dogs in the detailed epidemiological information collected in the study, suggesting that the increase in rabies VNAb titre is likely to be from a natural exposure to rabies virus. In a genuine anamnestic immune response the VNAb titre on secondary exposure would be expected to be greater than at primary vaccination (Zhang et al., 2011, Derbyshire and Mathews, 1984), 8/13 (62%) of these dogs had a higher VNAb titre at tp 6 than at tp 1 (two dogs were excluded from analysis as they did not have rabies VNAb titres measured at tp 1).

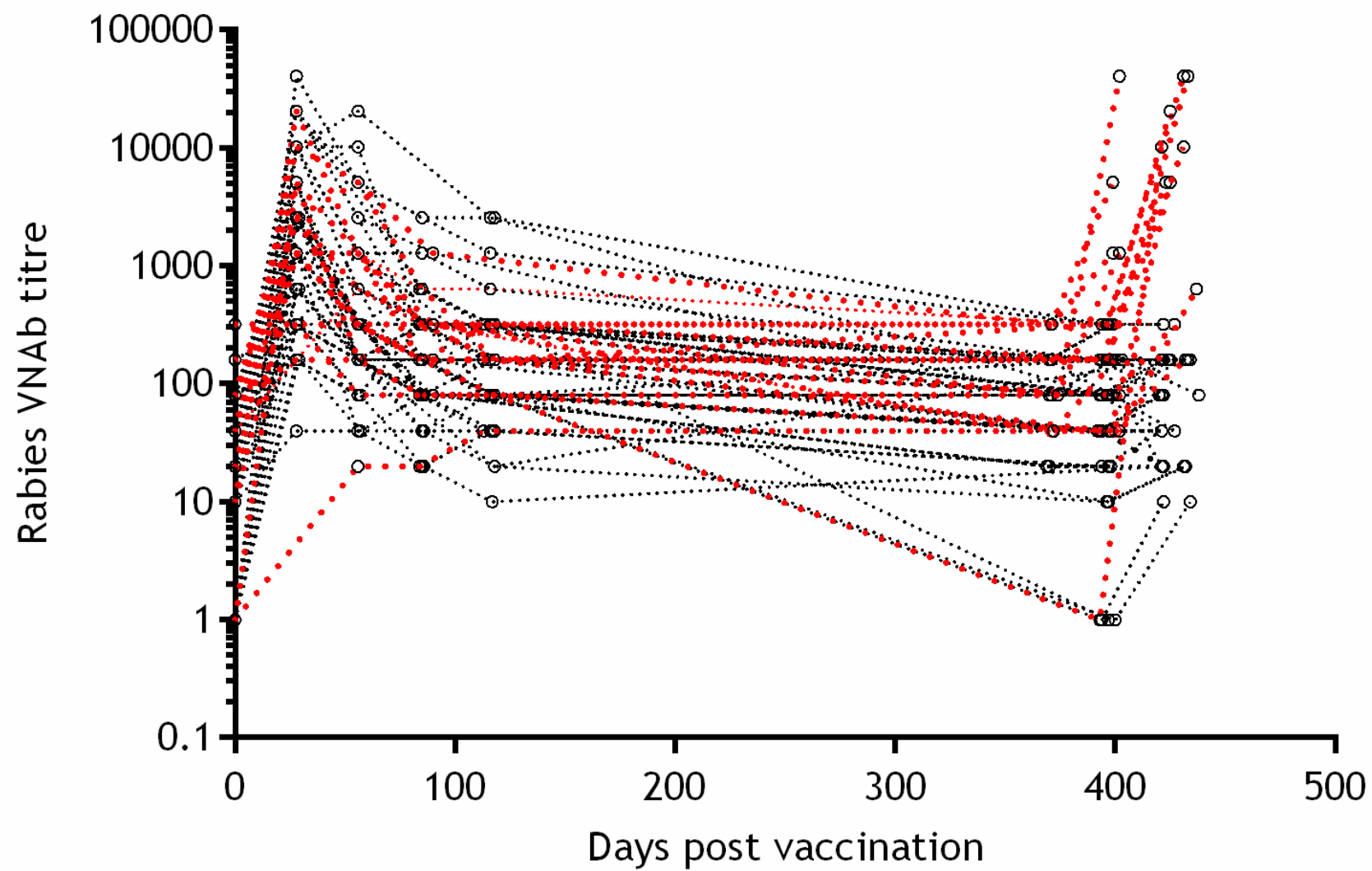


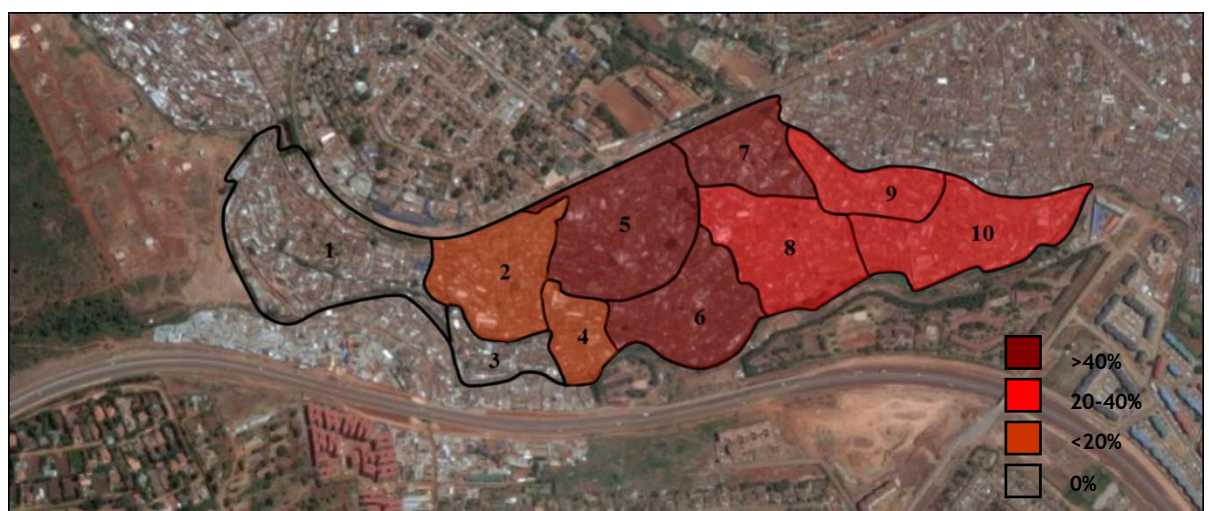
Figure 4.15 Rabies virus neutralising antibody titres of dogs from pre-vaccination to time point 6. Titres of all dogs (n=67) at each time point in the study (n=431). Individual dogs are represented by dashed lines, circles indicate day of sampling, dogs which had a four-fold or greater increase in titre between time point 5 and 6, and a titre of ≥ 40 at tp 6 are displayed by red dashed lines (n=15).

Dog ID	Age	Sex	Condition	Cluster	Multi-dog household	Time point 0 titre	Time point 1 titre	Time point 0-1 fold increase	Time point 5 titre	Time point 6 titre	Time point 5-6 fold increase
12	1-4 y	M	3	2	Y	80	1280	16	320	20480	64
57	4+ y	M	2	4	N	320	ND	-	160	5120	32
91	1-4 y	M	2	5	N	320	320	1	40	5120	128
116	Adult	M	3	10	N	40	2560	64	40	160	4
122	Adult	M	3	9	Y	<10	5120	5120	<10	10240	10240
148	4+ y	M	2	8	N	20	2560	128	160	≥40960	256
157*	4+ y	M	3	5	Y	10	1280	128	320	≥40960	128
158*	4+ y	M	3	5	Y	40	1280	32	160	10240	64
162	Adult	F	2	7	N	<10	ND	-	40	640	16
174	4+ y	M	2	6	Y	10	20480	2048	80	320	4
244	Adult	F	2	6	N	80	5120	64	320	1280	4
247	1-4 y	F	2	6	N	40	10240	256	320	5120	16
260	Adult	F	1	6	N	80	2560	32	40	160	4
265**	Adult	F	2	6	Y	160	320	2	80	1280	16
266**	Adult	F	2	6	Y	80	160	2	80	≥40960	512

Table 4.8 Characteristics of dogs with a four-fold or greater increase in rabies virus neutralising antibody titre between time point 5 and time point 6. Data for dogs which had a four-fold or greater increase in rabies VNAb titre between time point 5 (369-403 days post vaccination) and time point 6 (397-438 days post vaccination). Age represents the age category of the dog at time point 6. ND=not done. * and ** denote pairs of dogs from the same household.

Evidence of natural exposure to rabies virus in these dogs is strengthened by the clustering of these high titre dogs relative to their household location with three dogs in cluster 5, two dogs in cluster 6 and one dog from clusters 2, 8 and 9 (Figure 4.16). Additionally, two pairs of dogs (dogs #157 and #158, and dogs #265 and #266) who demonstrated an anamnestic response at tp 6 were from the same household, one pair from cluster 5 and one from cluster 6, possibly suggesting that these dogs were exposed to rabies antigen from the same source.

a)



b)

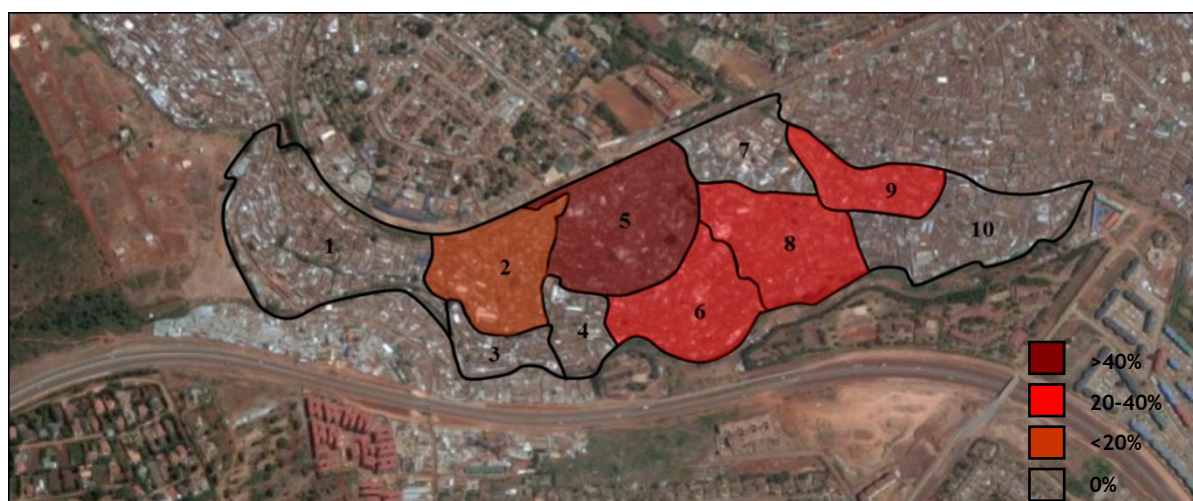


Figure 4.16 Heat maps of dogs with a four-fold or greater increase in rabies virus neutralising antibody titres between time point 5 and time point 6. Heat maps representing the household clusters of study dogs from the Kibera slum. a) Percentage of dogs per cluster with a four-fold or greater increase in rabies VNAb titre between time point 5 and 6 (15/60) b) Percentage of dogs per cluster with a four-fold or greater increase in rabies VNAb titre between time point 5 and 6, and a rabies VNAb titre greater at time point 6 than at time point 1 (8/13).

4.2.3.5 Cross-neutralisation of virus neutralising antibody elicited by rabies vaccination against different lyssavirus pseudotypes

Dogs in the study were vaccinated using a monovalent inactivated rabies virus vaccine (Rabisin, Merial). To investigate whether the VNAb elicited following vaccination would cross-neutralise other members of the *Lyssavirus* genus, eight serum samples collected 28 d post vaccination were tested for VNAb using six other MLV(lyssavirus) pseudotypes; LBV, MOKV, DUVV, EBLV-1, EBLV-2 and ABLV. Figure 4.17 demonstrates cross-neutralising activity of the dog serum against all phylogroup 1 lyssaviruses, with the VNAb titres to each lyssavirus generally correlating to the amino acid similarity of that virus to rabies virus (Table 4.9). All serum samples achieved a VNAb titre of ≥ 40 for all phylogroup 1 lyssaviruses

and all samples had a VNAb titre to the phylogroup 2 lyssaviruses (LBV, MOKV) of ≤ 20 , with the exception of one serum sample (#247) which had a VNAb titre of 40 to LBV. Dogs with the highest rabies VNAb titre generally had the highest VNAb titres to other phylogroup 1 lyssaviruses (Figures 4.17 and 4.18).

Amino acid similarity to rabies virus (%)		
Phylogroup 1	ABLV	68
	EBLV-2	67
	EBLV-1	63
	DUVV	61
Phylogroup 2	LBV	46
	MOKV	44

Table 4.9 Amino acid similarities between the ectodomain of the rabies virus glycoprotein and the ectodomain of six other lyssavirus species. The amino acid sequences of the strains of lyssaviruses used to produce the pseudotypes in this study were compared by pairwise analysis using a maximum composite likelihood model in MEGA6 (Tamura et al., 2013).

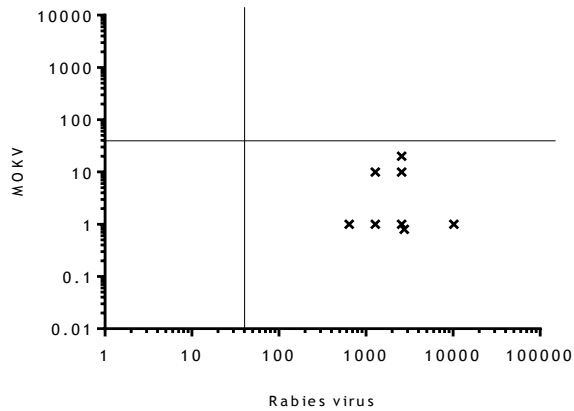
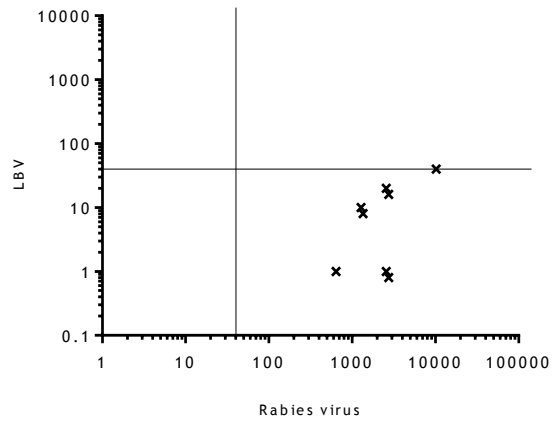
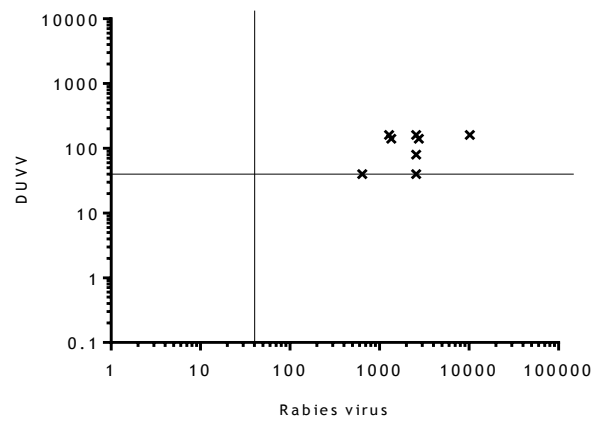
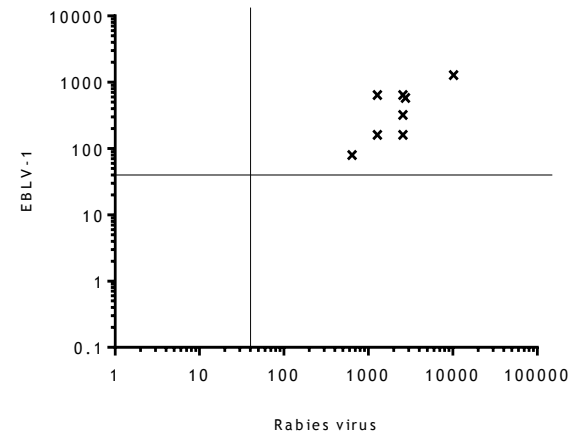
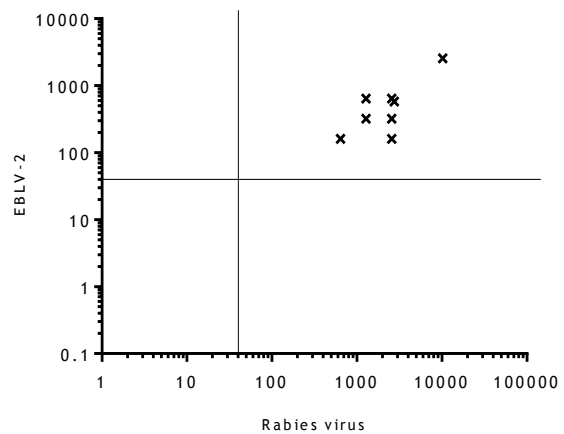
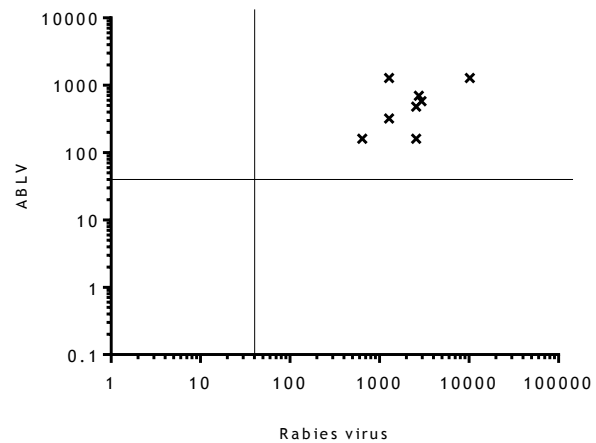


Figure 4.17 Comparison of rabies virus neutralising antibody titre and titres against other lyssaviruses in dogs vaccinated against rabies virus. Serum from dogs 28 d post rabies vaccination (n=8) were tested for VNAb against phylogroup 1 lyssaviruses: rabies virus, DUVV, EBLV-1, EBLV-2, ABLV; and phylogroup 2 lyssaviruses: LBV, MOKV. A small amount of random displacement in both axes has been added to the position of points to allow visualisation of overlapping data points. Solid line represents a titre of 40.

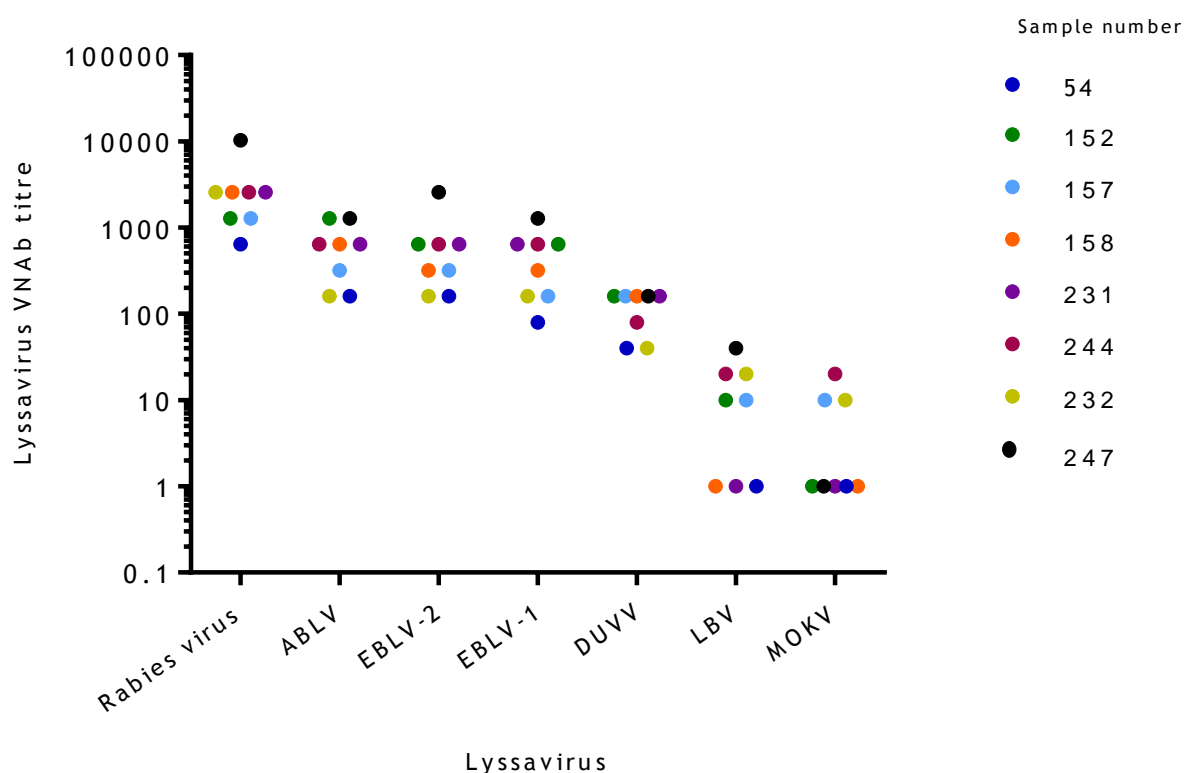


Figure 4.18 Virus neutralising antibody titre to 7 lyssaviruses in dogs vaccinated against rabies virus. Serum from dogs vaccinated against rabies virus was tested for VNAb at 28 d post vaccination (n=8). These samples were tested by pNT for VNAb against rabies virus, LBV, MOKV, DUVV, EBLV-1, EBLV-2 and ABLV. Lyssaviruses on X-axis are ordered by decreasing amino acid similarity to rabies virus as calculated in Table 4.9.

4.3 Discussion

The three serosurveys presented in this Chapter demonstrate the practical application of the pNT. In total we tested 705 samples from 18 different species for rabies VNAb. Only a small proportion of samples (n=9) tested were inconclusive by rabies virus pNT, all of which were wildlife samples. Inconclusive results were due to cytotoxicity, sample haemolysis or non-specific fluorescence. It is well documented in the literature that cytotoxicity and haemolysis commonly affect VNTs (Shiraishi et al., 2014a). Cytotoxicity affects the viability of the cell monolayer, pseudotypes therefore cannot infect cells and the pNT cannot be interpreted. Haemolysis is due to the degradation of erythrocytes, causing the release of haemoglobin and other cellular components into plasma which can cause non-specific inhibition of virus. Fragments of degraded erythrocytes can also obscure the cell monolayer in the pNT and haemolysed samples can negatively affect many laboratory assays, not only VNTs (Lippi et al., 2006). Haemolysis can occur *in vivo* or *in vitro* and is more common in wildlife samples due to poor quality serum being collected under challenging field conditions or when collected post-mortem (Boadella and Gortázar, 2011). Therefore visual inspection of serum samples should be performed routinely prior to testing using the rabies virus pNT.

A limitation of our assay is the lack of inclusion of the OIE reference serum, which is required to convert the highest dilution of serum achieving neutralisation into the internationally accepted unit for rabies VNAb titre (IU/ml). Our results are therefore recorded as simply the reciprocal of the highest dilution at which neutralisation occurred. It would have been preferable to have converted our titres to IU/ml to make our results directly comparable to other studies, this would be particularly useful regarding the measurement of cross-neutralising antibodies. The 0.5 IU/ml threshold is the titre considered protective in vaccinees but is itself a rather arbitrary value and warrants further critical discussion in Chapter 7. The VNAb titre employed as evidence of natural exposure to rabies virus is variable among studies, ranging from 0.06 IU/ml to 0.8 IU/ml (Ellison et al., 2014, Reynes et al., 2004). The Advisory Committee on Immunisation Practices suggest sera that completely neutralises rabies virus at a dilution of 1:5 should be considered positive for rabies-specific VNAb (ACIP, 1999), although non-specific inhibition at such low titres have been documented

in the literature (WHO, 2007, EFSA, 2006, Mani and Madhusudana, 2013). Therefore it has been suggested that a more conservative dilution of 1:25 should be considered positive (Gilbert et al., 2012). The results obtained from our study on the Russian wildlife sera support such a suggestion, with rabies VNAb titres of 1:20 proving negative when tested by the FAVN test. It is possible that the level of rabies VNAb present in natural exposure could be less than the threshold of 1:40 employed in this study but due to the possibility of false-positives we concluded a conservative cut-off of 40 was appropriate, as in these serosurveys we considered specificity to be more important than sensitivity.

There were no seropositive samples amongst the sera collected from the Russian Far East. No cases of rabies have been officially documented in the Primorsky Krai region of the Russian Far East in the last 10 years (Miquelle and Murzin, 2003), therefore the results presented in this study are consistent with these data. There was a fatal case of rabies caused by Irkut virus, a phylogroup 1 lyssavirus (Liu et al., 2013), in a girl in the region in 2007 after contact with a bat (Leonova et al., 2009). As cross-neutralisation across the phylogroup 1 lyssaviruses is well documented, knowledge of circulating lyssaviruses in the region would be an important consideration for interpretation of seropositive results. If we had detected positive serology in the Russian samples, the serum should have been further tested for neutralisation against any other circulating lyssaviruses, the virus with the highest VNAb titre would be assumed to be the primary inducing antigen. Such tests would be easy to perform on the pNT as the only difference in the protocol would be the alteration of the glycoprotein gene included during transfection.

The lion serology from the SNP, was in stark contrast to the negative serology of the Russian Far East, with an overall seroprevalence of 20%. Berentsen et al. (2013) described rabies VNAb titres in 37% of lions tested in Zambia and reported no clinical disease in these animals. In their study samples were tested by RFFIT using a threshold of 0.2 IU/ml as evidence of seropositivity, regardless of this low cut-off 63% of these lions had titres ≥ 0.5 IU/ml. Additionally, a further study screening 149 lions from Tanzania, Botswana and Zimbabwe, showed an overall seroprevalence of around 13% (Monique Léchenne, 2016, personal communication). Moreover, evidence of seroconversion to rabies virus in these lions was also reported from the same study. The results of our study suggest a

proportion of lions in the SNP have been exposed to rabies virus, further contributing to evidence of rabies VNAb titres in African lions presented in previous studies. Lembo et al. (2008) report rabies cases in other wildlife species in the SNP in years correlating to the seropositive lions in our studies, further supporting these results. The route of virus exposure in lions is unknown and could be related to hunting infected prey, as lions typically suffocate or strangle their prey, bringing the lion's mouth and eyes in contact with infected saliva (Berry, 1993), or due to ingestion of infected prey. Alternatively, lions could become exposed due to social licking and biting as suggested by East et al. (2001) in hyenas, or indeed lions could be bitten by a rabid animal, which is the most common route of rabies virus infection.

The study of the cohort of dogs from Kibera was intended to provide in depth serological data on the VNAb response to rabies vaccination in free-roaming dogs in a rabies endemic setting. There have been previous studies regarding response to rabies vaccine, however most have focused on experimental dogs (Hu et al., 2008, Shiraishi et al., 2014b) or domestic animals from non-endemic communities (Berndtsson et al., 2011) and concerns have been raised over whether such immune responses would be mimicked in free-roaming dogs. In Africa where dogs are the primary reservoir of rabies, mass dog vaccination is critical for rabies elimination. It has been estimated that levels of only 20-45% vaccination coverage could be required to eradicate rabies (Hampson et al., 2009), this figure is highly achievable but in resource limited settings vaccination strategies must be used to the greatest effect. Our study aimed to provide data to improve evidence based vaccination policy in such settings. Morters et al. (2014a) have recently published a longitudinal serological study with cohorts similar to those presented herein, focusing on dogs from South Africa and Indonesia. The results of our longitudinal serosurvey support Morters' study, demonstrating an initial rapid increase in rabies VNAb at seroconversion, a fairly swift decline to around day 60 and then a further gradual decline throughout the study.

Our results demonstrate that all immunised dogs mount a protective antibody response to rabies at 4 weeks post vaccination regardless of age, sex or condition at vaccination. Although all dogs had a protective titre, there was significant variation in the magnitude of response to vaccination, we

investigated the source of this variation using statistical models. Our results complement previous studies conducted on rabies vaccine response demonstrating that sex and age are not significant factors in rabies vaccination response (Tepsumethanon et al., 1991, Morters et al., 2014a, Berndtsson et al., 2011). Our models suggest that a small amount of variation is attributed to the body condition of the dogs at vaccination, with dogs of poorest condition responding significantly less effectively than dogs with a condition score of 2 or 3. However, the majority of the variation in the magnitude of vaccine response could be attributed to the pre-vaccination titre, with a high pre-vaccination titre being negatively correlated to the magnitude of response. We cannot find any other studies which consider the pre-vaccination rabies VNAb titre in vaccine naive dogs as an attributing factor to vaccine response, presumably as all vaccine naive dogs are assumed to be serologically negative. However, studies of bacterial vaccines have also reported a significant inverse relationship between pre-vaccination titre and antibody response (Hall et al., 2001).

At one year post primary vaccination the majority of dogs retained a rabies VNAb titre of ≥ 40 . It has been suggested that rabies VNAb drops rapidly to insufficient levels in animals who do not mount an efficient initial vaccination response. Our models demonstrated that although the peak VNAb titre in these dogs accounted for a very small proportion of the variation in titre at one year, when this result was considered a binary value of 'protected' or 'not protected' there was no significant relationship. This observation is important when considering population coverage achieved in vaccination campaigns. Further studies of persistence of VNAb following booster vaccination would also be valuable data to consider in such a cohort.

Although 0.5 IU/ml is considered the threshold of protection, many rabies challenge studies have reported previously vaccinated dogs with rabies VNAb titres below this threshold surviving challenge, whilst unvaccinated controls almost always succumb to the disease (Sikes et al., 1971, Brown et al., 1973). A challenge study by Lawson and Crawley (1972) demonstrates survival of dogs vaccinated five years previously despite 50% of these dogs having undetectable antibodies prior to challenge. Hence the rate of protection in our dog cohort at one year post vaccination is likely to be an underestimate of true protection.

The work presented in this Chapter also demonstrates the utilisation of the pNT for non-rabies lyssaviruses, demonstrating the ability to measure neutralising activity to 7 different lyssaviruses. Our results support previous other studies demonstrating that vaccination with rabies virus elicits antibodies with cross-neutralising activity against the other phylogroup 1 lyssaviruses but not against phylogroup 2 lyssaviruses (Reynes et al., 2004, Malerczyk et al., 2009, Wright et al., 2008, Badrane et al., 2001). There is no VNAb titre considered protective for non-rabies lyssaviruses but if we employ the accepted protective titre for rabies virus of 0.5 IU/ml (and the pNT equivalent cut-off of 40), all dogs tested would have an adequate VNAb response to protect from challenge with phylogroup 1 lyssaviruses at 28 d post rabies vaccination. The level of neutralisation against each lyssavirus correlated to the amino acid sequence homology between the tested lyssavirus and rabies virus, demonstrating increased protection against the viruses with increasing genetic similarity to rabies virus, such antigenic relationships were first described by Badrane et al. in 2001. These results strengthen the recommendations that individuals at risk of non-rabies lyssaviruses, such as bat handlers, are vaccinated with rabies vaccine and treated with rabies post-exposure immunoglobulin if a high risk exposure occurs (PHE, 2013). Protection against EBLV-2, a phylogroup 1 lyssavirus, is of particular importance in the UK as in 2002 there was a fatal case of EBLV-2 in Scotland in an unvaccinated bat handler (Nathwani et al., 2003), and EBLV-2 seropositivity has been reported in Scottish bats (Brookes et al., 2005a). As the VNAb activity to other phylogroup 1 lyssaviruses is less effective than for rabies virus, boosters may be required more frequently and further studies are necessary to determine the kinetics of rabies vaccination response to these lyssaviruses. Although the majority of sera from dogs immunised against rabies failed to neutralise the phylogroup 2 lyssaviruses one dog sample neutralised LBV at a titre of 40, this dog had the highest rabies VNAb titre (10240) of all dogs tested in the cross-neutralisation experiment. Cross-neutralisation of LBV by rabies VNAb has also been documented in previous studies, especially when high VNAb against rabies are present (Morters et al., 2014a, Hanlon et al., 2005). Similar studies have also shown a low level of cross-neutralising activity of rabies VNAb against MOKV (Hanlon et al., 2005). There are no biologics designed specifically for phylogroup 2 lyssaviruses, with the absence of any therapeutic agents for these viruses rabies vaccination and post-exposure immunoglobulins may be given in the hope

that a considerable immune response ensues and some cross-protection occurs. More research is required to further understand cross-neutralisation of non-phylogroup 1 lyssaviruses and the pNT proves a good candidate for such experiments as they can be performed in any BSL-2 laboratory, at low cost, without risk to personnel.

In addition to measuring the immune response to vaccination, we also measured pre-vaccination rabies VNAb titres in our dog cohort as a baseline to calculate the increase in titre between pre- and post-vaccination samples. Over half the dogs in our cohort had a pre-vaccination rabies VNAb titre of ≥ 40 , which would be considered a protective antibody titre. Reports of rabies VNAb in previously unvaccinated dogs have also been documented in other serological studies from rabies endemic populations (Morters et al., 2014a, McNabb, 2008, Cleaveland et al., 1999). To further confirm the specificity of our assay we tested 30 serum samples from UK dogs and all but one sample gave the lowest recordable titre on the pNT. Dogs born in the UK are not the best control group, as these dogs are generally well cared for, non free-roaming and not in contact with the plethora of infectious diseases to which slum dogs are exposed. However, gaining serum samples from similar slum dogs from a rabies free location, although preferable, was unachievable. One of the major advantages of the pNT over the FAVN is that each sample is simultaneously tested for non-specific neutralising activity using the VSV control, hence we are confident that these pre-vaccination titres are genuine and not due to non-specific neutralisation.

There are various potential explanations for the relatively high pre-vaccination titres observed in this study which include previous vaccination, detection of cross-neutralising antibodies, an inappropriate cut-off titre for use in this population or finally a genuine, natural past exposure to rabies antigen. The authors of the initial study are confident there were no previous rabies vaccination campaigns at the Kibera study site and as these dogs are slum dogs it is very unlikely they would have been privately vaccinated. The detection of cross-neutralising antibodies is a possible explanation for these pre-vaccination positives, and Duvenhage virus has been isolated from bats in Kenya (Van Eeden et al., 2011). Alternatively, the cut-off of 40 used in this study could be too low for this dog population, as the cut-off of 0.5 IU/ml is designed to demonstrate a

protective antibody titre post-vaccination in domestic dogs from non-endemic settings.

A further possibility to consider is the potential for these dogs to have been naturally exposed and to have seroconverted to rabies virus. As the dogs were alive and well for at least one year after sampling, if natural infection had occurred it appears to have been without significant clinical symptoms. Such abortive infections are an accepted phenomenon of rabies infection in bats (Turmelle et al., 2010) and have also been demonstrated in humans (CDC, 2010) and experimentally infected mice (Vanag et al., 1981). In previously exposed dogs an anamnestic response would be expected upon vaccination, and this has been documented previously in Tanzanian dogs with low titres following presumed natural exposure (Cleaveland, 1996). However, as demonstrated in the general linear model this was not the case in all dogs in this study, with the five dogs not achieving a four-fold increase in titre at tp 1 all having pre-vaccination titres of >80. However, vaccination after natural exposure to rabies virus has not been studied and the response to re-exposure may be atypical, such atypical reactions have been observed in other RNA virus infections (Murtaugh and Genzow, 2011).

Further evidence of natural exposure to rabies occurred at the final sampling point in the study, where an increase in VNAb titre was observed in a high proportion of the dog cohort, possibly indicating viral exposure. A four-fold increase in titre was seen at initial vaccination and tp 6 only, so this is unlikely to be an artefact of the assay or natural fluctuation in VNAb titres. There were no other known rabies vaccination campaigns during the study period and there was no record of rabies vaccination in the detailed epidemiological data collected along with the samples, therefore these responses are highly unlikely to be vaccine induced. The majority of these dogs demonstrated an anamnestic response however 38% did not, although as the date of any possible exposure is unknown we may have sampled at the start of the secondary immune response. The geographical clustering of the high titre dogs is also indicative of a central source of exposure, in addition to two pairs of dogs from the same households both experiencing increased titres. It would have been of interest to continue monitoring the antibody response in these dogs and to determine whether they were protected from disease, especially as one dog had an undetectable VNAb

titre at tp 5. The authors of the original study were not aware of any formal reports of rabies cases in Kibera during the study period, however this may be due to a lack of reporting or because all exposed dogs were protected through vaccination.

A major finding in this Chapter is the evidence of rabies VNAb in both healthy lions and unvaccinated dogs, from rabies endemic settings. The significance of these results requires critical discussion. As previously mentioned rabies VNAb titres have been previously reported in unvaccinated dogs and wildlife, with VNAb to non-rabies lyssaviruses being reported in bat species globally (Brookes et al., 2005a, Hayman et al., 2008, Arguin et al., 2002, Pérez-Jordá et al., 1995, Markotter et al., 2013). Non-fatal rabies infection is an accepted outcome of rabies infection in bats, but such abortive infection in other species challenges the central theory that rabies infection is always fatal in terrestrial mammals. Gilbert et al. (2012) have described rabies VNAb in bat-exposed communities in Peru suggesting non-lethal rabies infections in humans. The value of titres in their study ranged from 0.1-2.8 IU/ml with most measuring around 0.5 IU/ml, however the majority of these samples were negative when tested for rabies ribonucleoprotein IgM and IgG by the fluorescent antibody test. Cleaveland et al. (1999) also reported high rabies VNAB titres in unvaccinated dogs in Tanzania when tested by RFFIT, but no detectable antibody when tested by nucleoprotein ELISA. The level of neutralising antibodies to rabies is considered the best correlate of protection (Johnson et al., 2010), therefore when measuring vaccination response VNTs are the method of choice. However, these previous studies may suggest that measuring rabies antibodies directed against the nucleoprotein may be a more specific indicator of natural rabies exposure than the neutralisation assay. In relation to our studies, if time and cost were not an issue, it would have been worthwhile re-testing the VNAb positive lion sera and the positive pre-vaccination dog sera on an alternative assay. Additionally for poor quality samples, such as some of the wildlife samples tested here, an ELISA is considered a superior alternative to neutralisation tests, as it is less affected by poor quality sera (Cliquet et al., 2000) and also requires a lower volume of sera, which in wildlife testing is preferable.

The results reported in this Chapter demonstrate the practical applicability of the rabies virus pNT as a robust alternative to live virus neutralisation tests. The

wildlife serosurveys presented demonstrate the valuable results that can be obtained using the pNT, without relying on expensive assays which are restricted to very few specialised laboratories. Increasing the availability of these assays will undoubtedly improve our understanding of the ecology of rabies virus. The applicability of the pNT is increased by being easily adapted to measure VNAb to any of the lyssaviruses, demonstrated here using vaccinated dog sera. Furthermore, the results obtained in these three serosurveys gave results consistent with similar studies reported in the literature in which live virus neutralisation assays were employed.

Chapter 5. Novel approaches to detecting neutralising antibodies to Rift Valley fever virus

5.1 Introduction

Rift Valley fever virus (RVFV) is an arbovirus from the *Bunyaviridae* family. RVFV causes a disease which predominantly affects ruminants, however it is also an important zoonotic infection. Although most RVFV infections in humans are asymptomatic (Ikegami and Makino, 2011), severe illness can occur which may progress to haemorrhagic fever and death (Peters et al., 1989, Anyangu et al., 2010). In recent RVFV outbreaks human mortalities have reached several hundred (Kebede et al., 2010). The gold standard method for assessing exposure to RVFV is through detection of virus neutralising antibodies (VNAb) (OIE, 2012a). The virus neutralisation test (VNT) used for detecting RVF VNAb is the plaque reduction neutralisation test (PRNT). This assay requires test serum to be incubated with RVFV which is then incubated with a susceptible cell line and after 3-5 days the cell monolayer is examined for viral plaques. As RVFV is classified as a hazard group three pathogen, the PRNT must be performed at biosafety level 3 (BSL-3) (HSE, 2013). The lack of availability of the PRNT in RVFV endemic settings hampers its use for serosurveillance of RVFV, both during outbreaks and also during inter-epidemic periods. The use of enzyme linked immunosorbent assays (ELISA) is favoured for large scale serosurveillance of RVFV, however these assays are host species-specific and lack the ability to distinguish the many viral species within the *Phlebovirus* genus.

The aim of this study was to generate RVFV pseudotypes and to use these to replace live RVFV in a pseudotype neutralisation test (pNT). However, pseudotypes of RVFV were produced to very low titres and although we were able to demonstrate specific neutralisation of these pseudotypes by RVFV

specific VNAb, the large volumes of pseudotype required for the assay made it impractical. As an alternative to the pNT we developed a neutralisation test utilising a recombinant *Renilla* luciferase expressing RVFV, which allows for specific, rapid and high-throughput screening for RVF VNAb in the serum of any host species.

5.2 Results

5.2.1 Production of retroviral pseudotypes bearing the glycoproteins of Rift Valley fever virus

After the success of the rabies virus pNT we attempted to produce RVFV pseudotypes using the same methodology. Although a large number of viruses have been pseudotyped, including some bunyaviruses (Higa et al., 2012, Cifuentes-Muñoz et al., 2010, Tani, 2014, Ma et al., 1999), on commencing this study there were no published accounts of RVFV pseudotypes or pseudotypes of any of other phlebovirus to our knowledge. Hofmann et al. (2013) have since described the production of pseudotypes of severe fever with thrombocytopenia virus (SFTSV), a newly emerged member of the *Phlebovirus* genus and Bukbuk et al. (2014) have described the development of a RVFV pseudotype neutralisation test.

Phleboviruses are enveloped tripartite, single stranded RNA viruses, comprising of Large (L) Medium (M) and Small (S) segments. The M segment encodes two glycoproteins; Gn and Gc, in addition to two non-structural proteins; NSm1 and NSm2 (Kakach et al., 1989) (Figure 5.1). These proteins are produced from a single open reading frame by alternate use of the five initiation codons upstream of Gn (Ikegami, 2012). We amplified five sections of the RVFV M segment, from a plasmid encoding the entire cDNA sequence of the M segment of the MP-12 strain of RVFV (pTVT7R-GM). The five sections amplified were; the complete M segment, GnGc, NSm1, Gn and Gc. As pseudotyping bunyaviruses had not been previously attempted in our laboratory, we were unsure which of these proteins would be optimal for pseudotype production. Studies using a retroviral vector system to pseudotype Andes virus (ANDV) utilised the entire M segment for optimal pseudotype production (Cifuentes-Muñoz et al., 2010), however

Takehara et al. (1990) reported low expression of RVFV proteins when the entire M segment was expressed by recombinant baculovirus, but high expression when Gc was individually expressed. All five sections were cloned into the mammalian expression vector VR1012 and plasmids were designated RVF-M, RVF-GnGc, RVF-NSm1, RVF-Gn and RVF-Gc.

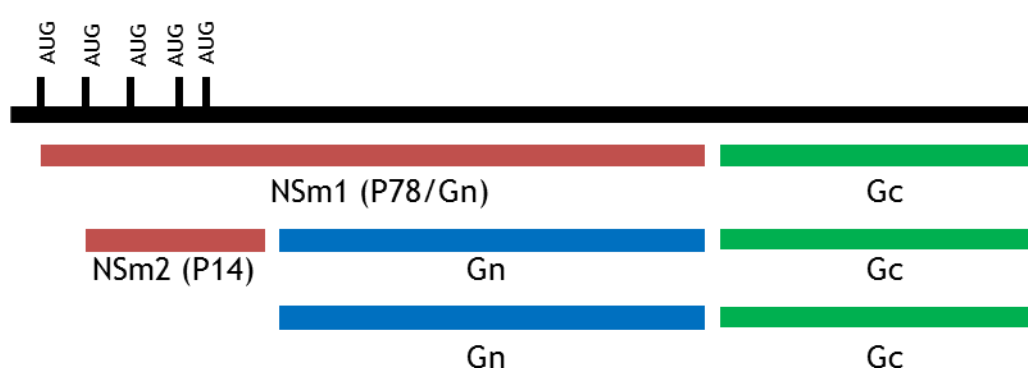


Figure 5.1 Schematic representation of mRNA transcription from the M segment of Rift Valley fever virus. Schematic diagram of the antigenomic M segment of RVFV mRNA and the proteins produced via alternate use of the five initiation codons. Gaps between segments represent post-translational cleavage sites. Figure adapted from Kreher et al. (2014).

Pseudotypes were produced using the method previously applied to MLV(lyssavirus) pseudotype production, with co-transfection of the MLV gag-pol packaging plasmid; CMVi, the GFP expression construct; CNCG, and the RVFV glycoprotein expression constructs. BHK-21 and Vero cells are widely used cell lines of choice for RVFV culture; hence we used these two cell lines for transduction of the MLV(RVFV) pseudotypes. As with all previous experiments a plasmid containing the glycoprotein of vesicular stomatitis virus (VSV) and an empty VR1012 vector (Δ Env) were transfected and transduced in parallel as positive and negative controls. Cells were examined for GFP expression at 24 h,

48 h and 72 h post transduction using fluorescence microscopy. Little GFP expression was observed in any cell line transduced with MLV(RVFPV) pseudotypes at any time point, however positive control MLV(VSV) pseudotypes were produced to a high titre (Figure 5.2). The only MLV(RVFPV) pseudotype to demonstrate GFP positive cells following transduction was the pseudotype produced using the GnGc polyprotein plasmid (RVF-GnGc), however only very few fluorescent cells were detected and only when transduced into Vero cells (Figure 5.2). The number of GFP expressing cells was not considered high enough to warrant analysis by flow cytometry, hence only visual inspection was noted.

To confirm RVFPV glycoprotein expression during transfection, Western blots were performed to detect protein expression in both cells transfected with RVF-GnGc and the resulting pseudotypes generated. A protein of approximately 54 kDa correlating to Gn was expected (Kreher et al., 2014). Cell lysate of RVFPV infected A549 cells were included in the Western blot as a positive control (Figure 5.3). A band matching the expected size for Gn observed in RVF-GnGc transfected 293T cells (lane 1) and also in the A549 positive control (lane 5), and was not present in the cell lysate or supernatant of cells transfected with an empty vector (lanes 3 and 4). A slightly larger band was observed in the concentrated MLV(RVFPV) pseudotype (lane 2) and was initially thought to correspond to RVFPV glycoproteins, however non-specific bands of the same size have been observed in supernatants of cells transfected with both rabies virus glycoprotein and empty plasmids (Figure 3.2). Therefore we concluded that RVFPV glycoproteins were being expressed in transfected cells however were not being readily incorporated into the retroviral pseudotype particles.

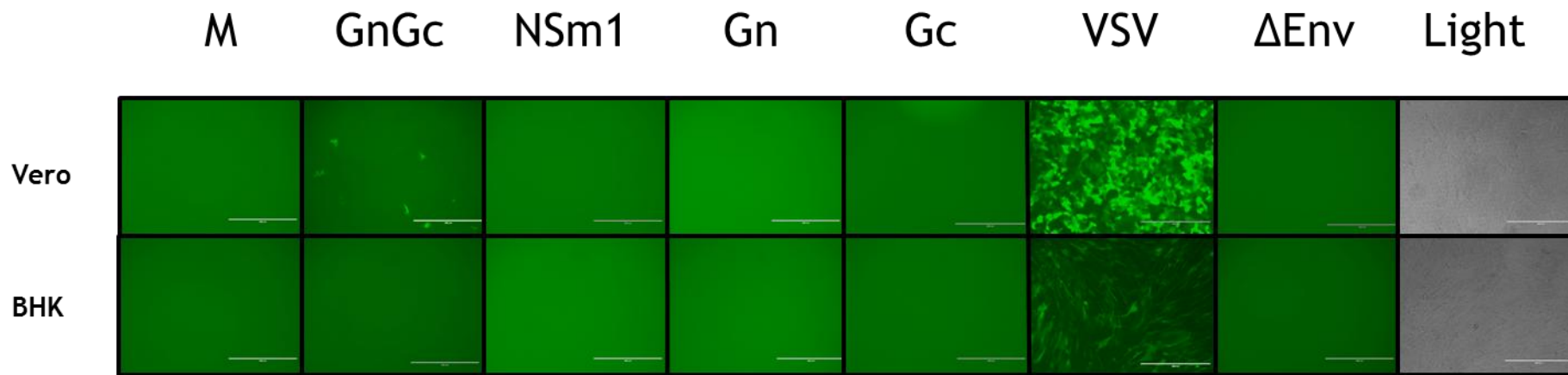


Figure 5.2 Transduction of Vero and BHK-21 cell lines with prospective Rift Valley fever virus pseudotypes. Undiluted supernatants bearing prospective MLV(RVFPV) pseudotypes were plated onto Vero and BHK-21 cell lines and visually inspected for fluorescence at 48 h. MLV(RVFPV) pseudotypes were produced using M, GnGc, NSm1, Gn and Gc segments; VSV and Δ Env were used as positive and negative control pseudotypes respectively.

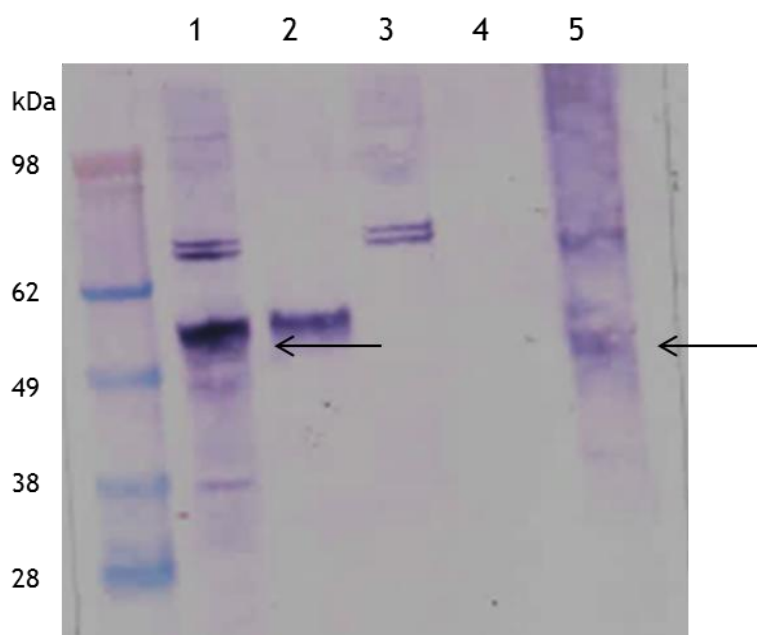


Figure 5.3 Expression of Rift Valley fever virus glycoproteins in pseudotypes and producer 293T cells. Western blot analysis of RVFV glycoproteins in 293T cells transfected with RVF-GnGc to produce MLV(RVFV) pseudotypes (lane 1), and the resulting RVFV concentrated pseudotype particles (lane 2). Cell lysates of 293T cells transfected to produce Δ Env pseudotypes (lane 3) and the resulting Δ Env concentrated pseudotype particles (lane 4). Cell lysate of A549 cells infected with the MP-12 strain of RVFV at 72 h (lane 5) was utilised as a positive control. Membranes were probed with mouse anti-Gn monoclonal antibody (4D4). Arrows represent bands specific for RVFV glycoprotein.

Various methods to improve the titre of the MLV(RVFV) pseudotypes were employed and in each experiment only the MLV(RVFV) pseudotypes produced using the RVF-GnGc expression construct expressed GFP following transduction of the target cells. These improvements included trialling different plasmid ratios during transfection, the optimum being a ratio of 1:1.5:1 of CMVi, CNCG and glycoprotein expression construct respectively. We also examined transfection with plasmids encoding Gn and Gc independently (RVF-Gn and RVF-Gc) in a four plasmid transfection (CMVi + CNCG + RVF-Gn + RVF-Gc), however no

GFP expression was detected following transduction, mirroring the results observed when RVF-Gn and RVF-Gc plasmids were transfected individually. In addition, we screened a variety of different target cell lines for pseudotype transduction and of these only Vero and CRFK cells were permissive to MLV(RVFPV) pseudotypes. Furthermore, we also attempted to concentrate the pseudotypes by ultracentrifugation, however this did not improve the pseudotype titre significantly.

As the GnGc pseudotypes were the only pseudotypes that produced GFP expression upon transduction, the RVF-GnGc expression construct was used in all future experiments to produce MLV(RVFPV) pseudotypes. In attempts to improve the yield of MLV(RVFPV) pseudotypes, transfections were carried out using a variety of construct combinations, as demonstrated in Table 5.1, as previous studies have demonstrated improved pseudotype production for individual viral glycoproteins with particular vector backbones (Carbonaro Sarracino et al., 2014, Cifuentes-Muñoz et al., 2010, Sandrin and Cosset, 2006). Pseudotypes derived from HIV-1, FIV and MLV were produced with various reporters including *lacZ*, DsRed and luciferase, in addition to GFP, to assess whether any improvement in reporter expression would be observed in transduced cells. Pseudotypes were transduced into Vero and CRFK cells and assessed at 48 h post transduction. No GFP expression was observed in cells transduced with FIV-derived pseudotypes. In HIV-1-derived pseudotypes bearing GFP and DsRed, a small number of reporter expressing cells were observed in the transduced cells, however there were also positive cells seen when HIV-1-ΔEnv pseudotypes were transduced in CRFK cells. Our laboratory and others (Kahl et al., 2004, Cifuentes-Muñoz et al., 2010) have experienced poor transduction efficiency of HIV-1-derived pseudotypes in Vero cells. Hofmann et al. (1999) have suggested this may be due to an intracellular inhibitor to HIV-1 in Old World monkey derived cell lines. As optimal transduction of RVFPV pseudotypes appeared to occur in Vero cells, the HIV-1-derived pseudotypes were therefore discounted for production of RVFPV pseudotypes. All RVFPV pseudotypes produced using luciferase as the reporter gene demonstrated a similar level of luciferase expression to that of the negative control (ΔEnv), as demonstrated in Figure 5.4, and were therefore not pursued any further.

Gag-pol vector	Reporter expression construct	Reporter gene	Viral origin
CMVi	CNCG	GFP	MLV
CMVi	pMFG	<i>lacZ</i>	MLV
Fb-luc		Luciferase	MLV
HIT60	CNCG	GFP	MLV
FP93	GinSin	GFP	FIV
FP93	LinSin	Luciferase	FIV
HIV-eGFP		GFP	HIV-1
HIV-luciferase		Luciferase	HIV-1
p8.2/p8.91	CSRW	DsRed	HIV-1
p8.2/p8.91	CSGW	GFP	HIV-1

Table 5.1 Retroviral constructs co-transfected with RVF-GnGc to produce Rift Valley fever virus pseudotypes. The gag-pol packaging and reporter plasmid combinations used, in addition to the plasmid encoding the RVFV glycoprotein sequence (RVF-GnGc), in attempts to produce functional RVFV pseudotypes.

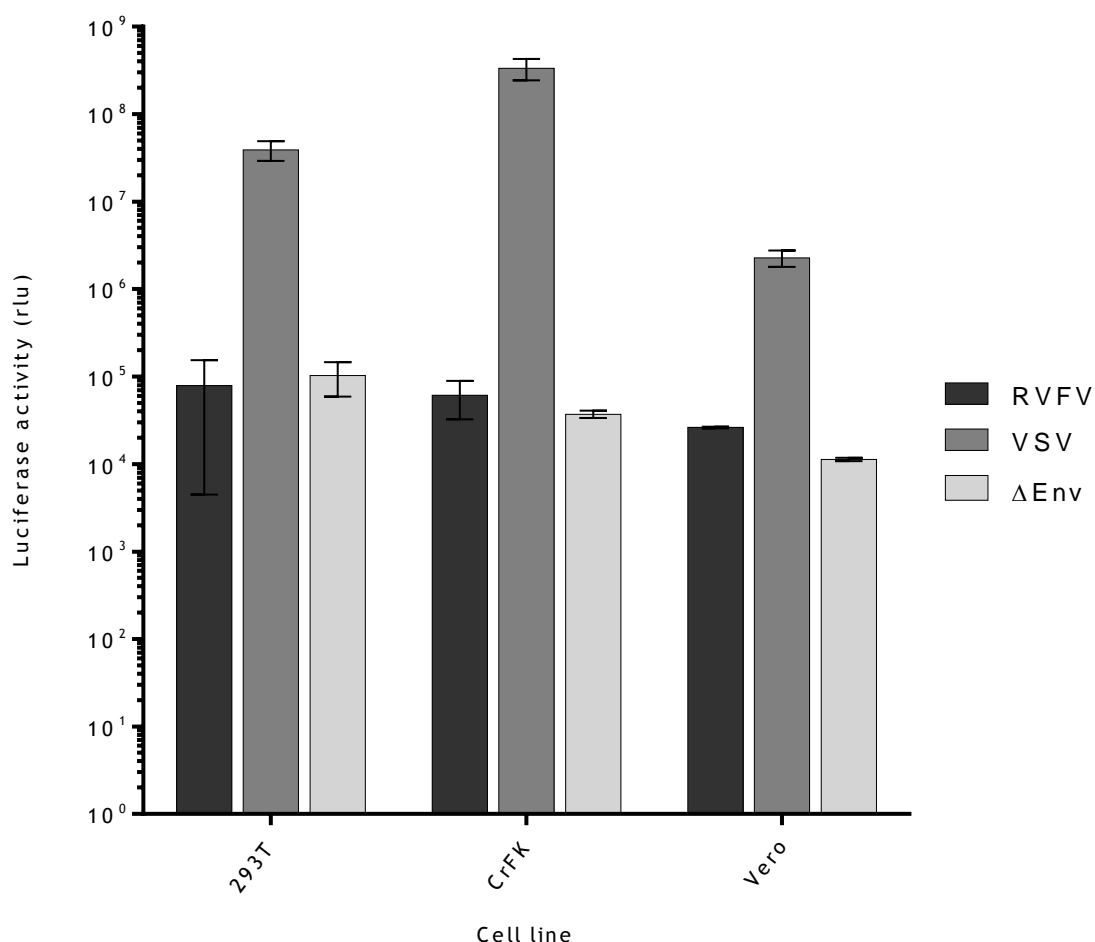


Figure 5.4 Luciferase expression at 48 h post transduction with Rift Valley fever virus and control pseudotypes. Undiluted pseudotypes were plated onto the specified target cell line and luciferase activity was measured at 48 h post transduction. Pseudotypes were produced using HIV-luciferase constructs co-transfected with the specified glycoprotein expression plasmid. The plasmid RVF-GnGc was used to produce MLV(RVFV) pseudotypes. MLV(VSV) and MLV(Δ Env) are the positive and negative control pseudotypes respectively.

After all attempts at optimisation, the highest titre of MLV(RVFPV) pseudotypes were produced by transfecting 293T cells with plasmids CMVi, CNCG and RVF-GnGc, while transducing either CRFK or Vero target cells (Figure 5.5). These transfections resulted in batches of pseudotype with titres of anywhere between approximately 50 and 1500 IU/ml. To determine whether the infectivity of these MLV(RVFPV) pseudotypes was due to genuine expression of RVFPV glycoprotein on the pseudotype surface, and not the result of ‘bald’ pseudotype particle infection (Voelkel et al., 2012), we investigated whether these MLV(RVFPV) pseudotypes could be neutralised by RVF VNAb. Two lion serum samples, one RVF VNAb positive and one negative, which had previously been tested by PRNT were used to determine whether MLV(RVFPV) pseudotypes could be neutralised. Briefly, neat MLV(RVFPV) pseudotypes were incubated with serial dilutions of test sera for 1 h at 37°C, the pseudotype/serum mixture was then added to Vero cells and incubated for a further 48 h. Cell monolayers were examined by fluorescence microscopy and the number of GFP expressing cells was counted manually. No GFP expression was observed when pseudotypes were incubated with the lowest dilutions of RVF VNAb positive sera. The number of GFP positive cells gradually increased as the concentration of serum decreased, suggesting that the MLV(RVFPV) pseudotypes were neutralised in a concentration-dependent manner by the VNAb present in the serum (Figure 5.6). GFP expressing cells were observed at all serum dilutions in the VNAb negative sample, although at dilutions of 1:5 and 1:10 numbers of GFP expressing cells were slightly lower than observed in the ‘no serum’ control well. A further five serum samples, collected from buffalo and previously tested for RVFPV antibodies by a RVFPV IgM capture ELISA, were tested for neutralising activity against MLV(RVFPV) pseudotypes. Samples were diluted to a final dilution of 1:100 and incubated with 100 µl undiluted pseudotype and matched concentrations of MLV(rabies) pseudotype as a control for non-specificity (VSV was not used as this virus is known to infect *Bovidae*). Three of the samples had tested positive and two negative by RVFPV ELISA. Both ELISA negative serum samples did not demonstrate neutralisation of the MLV(RVFPV) pseudotypes, two of the ELISA positive serum samples completely neutralised MLV(RVFPV) pseudotype, however one ELISA positive sample did not show neutralising activity against MLV(RVFPV) pseudotypes. All five samples showed no neutralisation against matched

concentrations of MLV(rabies) pseudotype, increasing evidence that the neutralisation observed was specific.

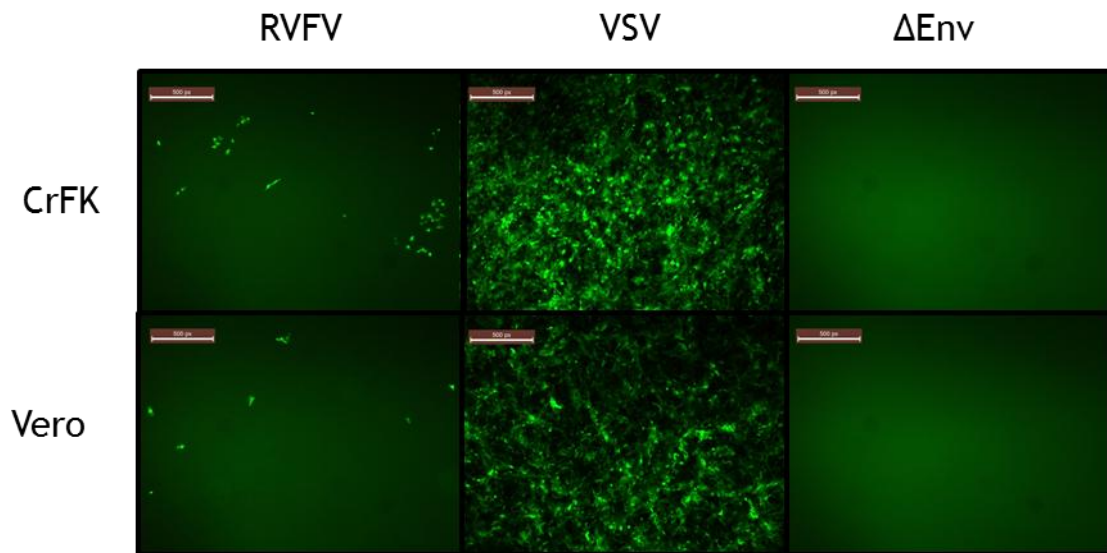


Figure 5.5 Rift Valley fever virus pseudotypes transduced into CRFK and Vero cell lines. Undiluted pseudotypes were transduced into CRFK and Vero cells, micrographs were taken at 48 h post transduction. Pseudotypes were produced by three plasmid transfection of CMVi, CNCG, and glycoprotein expression plasmid. MLV(RVFV) pseudotypes were produced using RVF-GnGc, MLV(VSV) pseudotypes were produced using VSV-G and MLV(ΔEnv) pseudotypes were produced using the empty plasmid vector VR1012. MLV(VSV) and MLV(ΔEnv) pseudotypes were employed as positive and negative controls respectively.

These results demonstrate the applicability of MLV(RVFV) pseudotypes as a substitute for live virus in a neutralisation test, however the titres of the MLV(RVFV) pseudotypes were considerably variable between batches and were significantly lower than those produced when pseudotyping lyssaviruses. Prior to use in a pNT, each batch of pseudotype requires the batch specific TCID₅₀ to be calculated to determine the concentration of pseudotype required in the assay.

Rough estimations based on average MLV(RVFPV) pseudotype titres suggest that if 100 TCID₅₀ were to be used in a RVFPV pNT, it would be necessary to use pseudotypes at neat concentrations, and depending on batch titre, with volumes possibly exceeding 100 µl. In the rabies virus pNT, over 1 ml of 100 TCID₅₀ pseudotype is used per sample, as samples are tested using doubling serum dilutions from 1:10 to 1:640 in triplicate. As titres of the MLV(rabies) pseudotypes are so high, the neat pseudotype is significantly diluted before use in the assay. If samples were tested in the same manner for a RVFPV pNT, a batch of MLV(RVFPV) pseudotype produced in a 10 cm dish, after calculation of TCID₅₀, would only be sufficient to test a handful of serum samples. Therefore although a RVFPV pNT is feasible; the low titre achieved using retroviral pseudotypes make such an assay laborious, costly and time consuming.

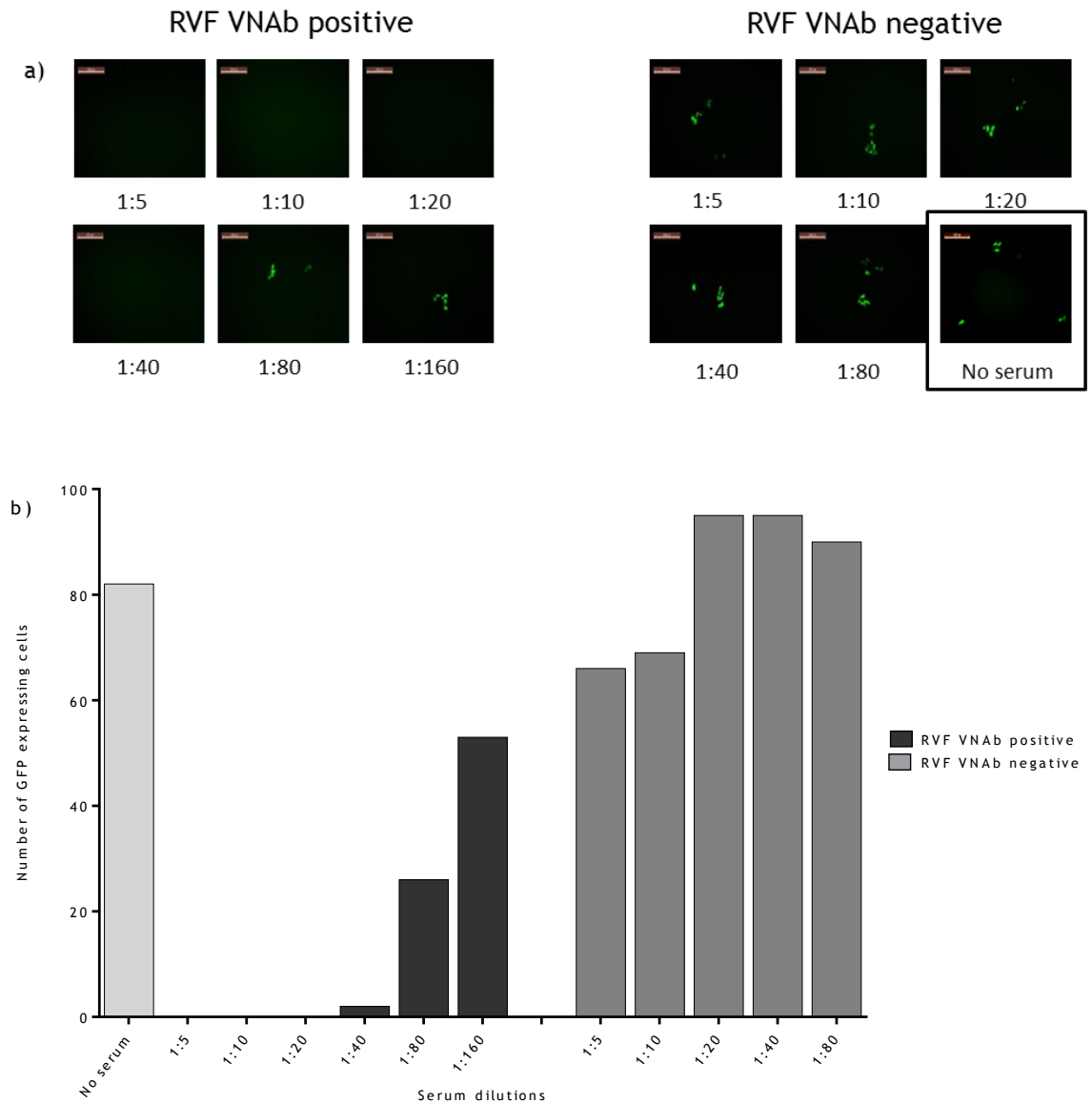


Figure 5.6 Neutralisation of Rift Valley fever virus pseudotypes by antibodies in lion serum. Lion sera, identified previously by PRNT as RVF VNAb positive or negative, were incubated with MLV(RVFV) pseudotypes to determine whether MLV(RVFV) pseudotypes could be neutralised by RVF specific VNAb. 100 μ l of neat MLV(RVFV) pseudotype was incubated with 100 μ l of serially diluted serum for 1 h at 37°C to allow neutralisation to occur, the pseudotype/serum mix was then added to Vero cells and incubated for a further 48 h, after which GFP expressing cells were counted manually by fluorescence microscopy. a) Fluorescence micrographs of wells containing MLV(RVFV) pseudotypes with increasing dilutions of serum, b) number of GFP expressing cells per well. The

'no serum' control contained 100 µl of culture medium in place of serum and was tested in parallel to the test samples.

In contrast to retroviruses, which assemble and bud at the plasma membrane, budding of bunyavirus particles occurs at the Golgi apparatus, the newly formed virions are then transported to the plasma membrane in Golgi derived vesicles (Nakitare and Elliott, 1993, Salanueva et al., 2003) (Figure 5.7). Sandrin and Cosset (2006) demonstrated that the intrinsic cell localisation of the glycoprotein expressed during pseudotype production, affected the incorporation of the glycoprotein into pseudotype particles. Previous studies have shown that mammalian plasmid expression of RVFV glycoproteins have resulted in non-detectable expression levels at the cell surface (Gerrard and Nichol, 2002, Filone et al., 2006). However, Filone et al. (2006) overcame this problem by saturating the Golgi retention mechanisms usually preventing transport of glycoproteins to the cell surface, by overexpressing GnGc using alphavirus replicons. In attempts to express the glycoproteins on the plasma membrane by overexpression of GnGc, we used a T7 polymerase/promoter system routinely used in RVFV research (Billecocq et al., 2008). A plasmid containing RVFV GnGc under the control of the T7 promoter (pTM1-GnGc) was kindly provided by Prof. Richard Elliott (University of Glasgow). The pTM1 vector is the expression plasmid of choice for many scientists working on RVFV as it provides high level protein expression (Billecocq et al., 2008). This vector requires the addition of T7 RNA polymerase (T7 pol) for expression, which can be supplied by transfecting the plasmid into a cell line that stably expresses T7 pol, or through co-transfection of a T7 pol-expressing plasmid. pTM1 was used in attempts to increase the expression of RVFV glycoproteins and subsequently improve the titres of MLV(RVFV) pseudotypes.

To determine whether the genes expressed in pTM1 plasmids would be expressed in BSR-T7/5 cells (a BHK-21-derived cell line stably expressing T7 pol) and in 293T cells in which T7 pol was provided *in trans* by co-transfection of a T7 pol expression plasmid, a pTM1 plasmid expressing GFP was transfected into both cell lines. A high level of GFP expression was observed in transfected BSR-T7/5

cells without the addition of a T7 pol plasmid, while GFP was expressed in 293T cells when T7 pol was provided *in trans* by plasmid co-transfection (Figure 5.8a). To investigate whether BSR-T7/5 cells could replace 293T cells for transfection, MLV(VSV) pseudotypes, which are always produced to a high titre, were produced in both BSR-T7/5 and 293T cells and the resulting pseudotype titres compared by transduction into CRFK cells. BSR-T7/5 cells did not successfully produce MLV(VSV) pseudotypes, there was very little GFP expression from MLV(VSV) pseudotypes produced in BSRT-7/5 cells, in contrast to those produced in 293T cells, which are the cell line of choice for pseudotype production (Figure 5.8b). Therefore BSRT7/5 cells were discounted as producer cells, however 293T cells could still be used for transfection using pTM1 plasmids when T7 pol was provided *in trans*.

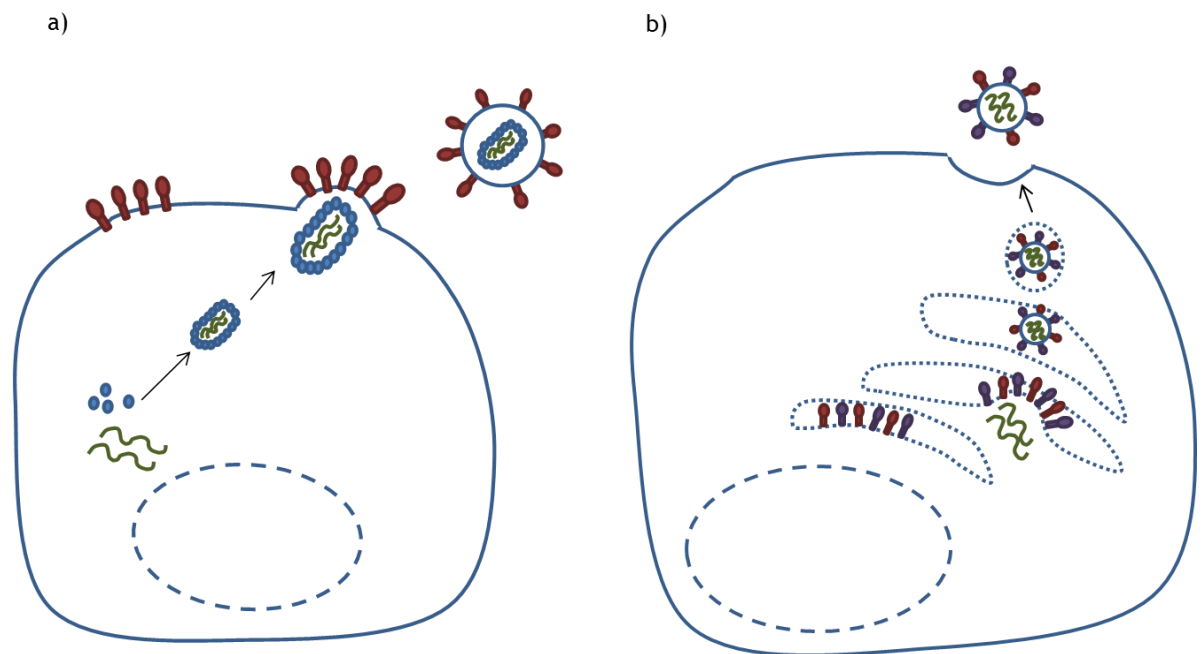


Figure 5.7 Schematic diagrams of retrovirus and bunyavirus budding. a) During retrovirus assembly, glycoproteins are expressed at the plasma membrane, the capsid protein is transported to the plasma membrane and the assembled virion pinches off. b) In contrast, during bunyavirus assembly the glycoproteins are expressed on the Golgi membrane, internal proteins are transported to the Golgi apparatus where virions assemble and bud into Golgi membrane derived vesicles, which migrate to the cell surface and fuse with the

plasma membrane for viral release. Figures adapted from Hutter et al. (2013) and Elliott (2014).

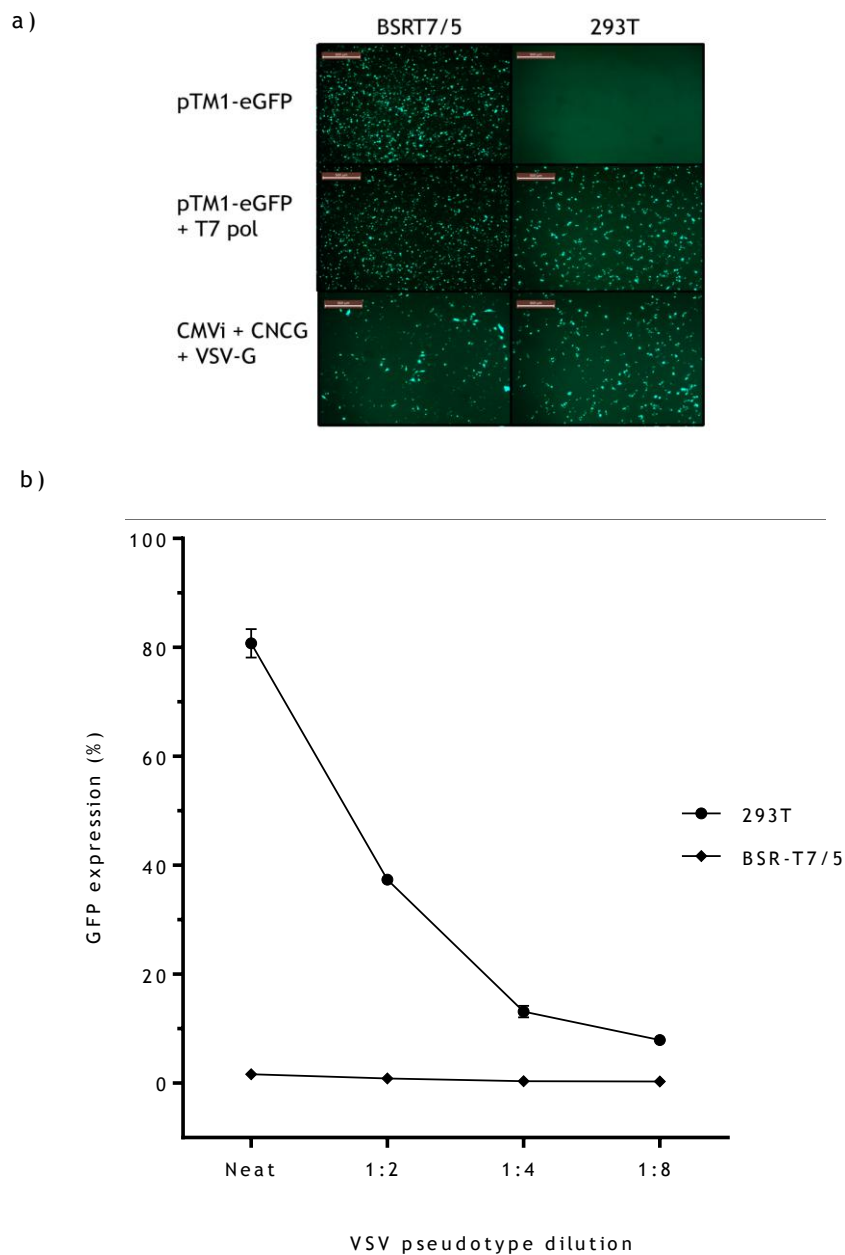


Figure 5.8 Expression of GFP in transfected BSR-T7/5 and 293T cell lines and titres of resulting MLV(VSV) pseudotypes produced in both cell lines. a) BSR-T7/5 and 293T cells were transfected with pTM1-eGFP, a GFP expression plasmid under the control of the T7 promoter, with and without co-transfection of a T7 pol expressing plasmid. Three plasmids; CMVi, CNCG and VSV-G, were co-transfected in both cell lines to produce MLV(VSV) pseudotypes. b) The resulting MLV(VSV) pseudotypes were then used to transduce CRFK cells where GFP expression was measured by flow cytometry.

To produce MLV(RVSV) pseudotypes using the T7 polymerase/promoter system, CMV, CNCG, pTM1-GnGc and T7 pol were transfected into 293T cells at varying ratios. Supernatants were harvested as previously described and were transduced into CRFK and Vero cells. A small number of GFP expressing cells was observed in wells transduced with MLV(RVSV) pseudotypes, however GFP expression was also noted in cells transduced with the negative control pseudotype (Δ Env). The Δ Env pseudotype was produced using an empty pTM1 vector in place of the pTM1-GnGc plasmid. The experiment was repeated using a new batch of 293T cells and GFP expression was still noted in the negative control wells. To determine if the GFP expression in the cells transduced with MLV(RVSV) pseudotypes was greater than in negative controls, transduced cells were analysed by flow cytometry. The GFP expression in RVSV transduced cells was of a similar magnitude to the negative control pseudotypes therefore we concluded that production of these pseudotypes was unsuccessful (Figure 5.9). To confirm expression of RVSV-GnGc from pTM1-GnGc, Western blot analysis was performed on cell lysates of cells transfected with CMV, CNCG, pTM1-GnGc and T7 pol and the resulting concentrated supernatant (Figure 5.10). As a negative control the same plasmids were transfected but lacking the T7 pol plasmid. As in Figure 5.3, a protein of the expected size (~54 kDa) was visualised in both cell lysates of pTM1-GnGc transfected cells and live virus infected cells, however no protein expression was observed in cell supernatants.

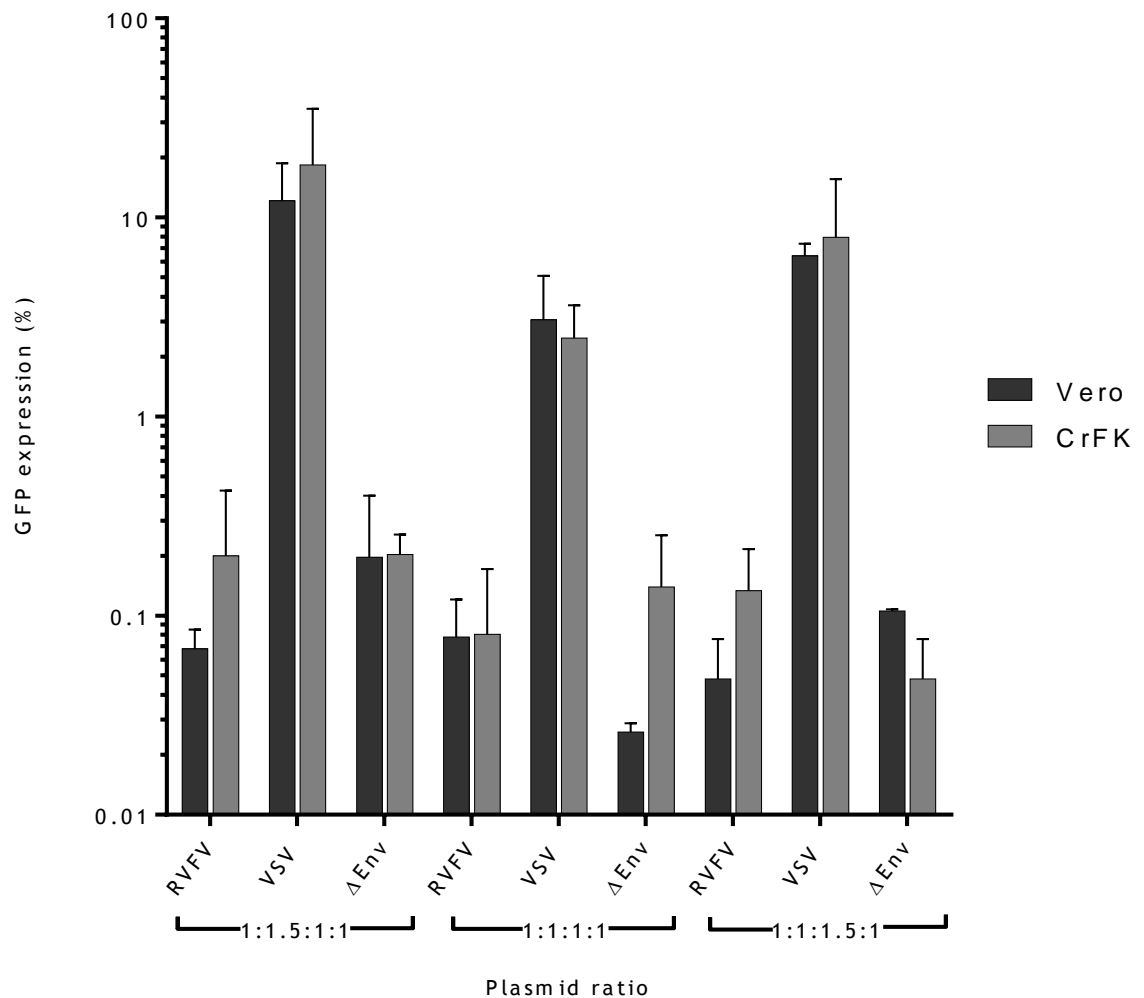


Figure 5.9 Expression of GFP in Vero and CRFK cells transduced with Rift Valley fever virus pseudotypes. Undiluted pseudotype was incubated with the specified cell line and GFP expression was measured by flow cytometry at 48 h post transduction. Pseudotypes were produced in 293T cells using CMVi, CNCG, pTM1-GnGc and T7 pol. X-axis displays the plasmid ratio of CMVi:CNCG:pTM1-GnGc:T7 pol respectively. VSV and ΔEnv are included as positive and negative control pseudotypes respectively.

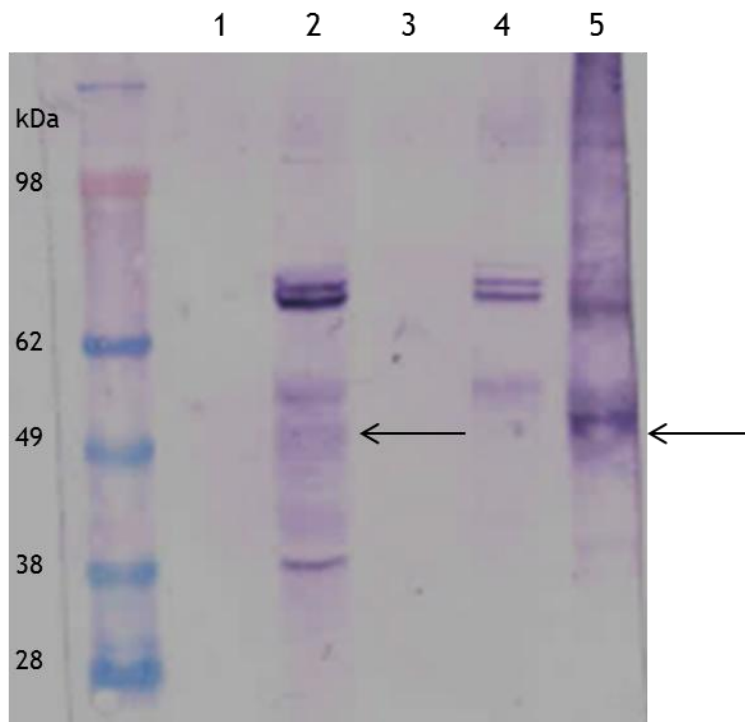


Figure 5.10 Expression of Rift Valley fever glycoprotein in pseudotypes and producer 293T cells. Western blot analysis of RVFV glycoproteins in RVFV concentrated pseudotype particles (lane 1), produced from 293T cells transfected with pTM1-GnGc with the addition of T7 pol (lane 2). As a negative control 293T cells were transfected as previously (lanes 1 and 2) but without the addition of the T7 pol plasmid, negative control concentrated supernatant (lane 3) and cell lysate (lane 4). Cell lysate of A549 cells infected with the MP-12 strain of RVFV at 72 h (lane 5) was utilised as a positive control. Membranes were probed with mouse anti-Gn monoclonal antibody (4D4). Arrows represent bands specific for RVFV glycoprotein.

Previous studies demonstrated increased expression of genes from the pTM1 vector when the transfected cells were co-infected with a recombinant vaccinia virus expressing T7 pol *in trans* (Elroy-Stein and Moss, 2001). To determine whether expression of GnGc could be increased from the pTM1 plasmid, this method was investigated. Transfections were performed as previously described using CMVi, CNCG and pTM1-GnGc plasmids and incubated for 4 h. Equal volumes of recombinant vaccinia virus and 0.5% trypsin were incubated at 37°C for 30 min, then added to the transfected cells and incubated for a further 24 h, pseudotypes were harvested as previously described. Pseudotypes were produced using different plasmid ratios to determine whether higher concentrations of pTM1-GnGc would improve pseudotype production, ratios of 1:1.5:1, 1:1:1 and 1:1:4 of CMVi:CNCG:pTM1-GnGc respectively were used in these experiments. Pseudotypes were transduced into BHK-21, Vero and CRFK cells. MLV(RVSV) pseudotypes produced using the vaccinia expression system produced pseudotypes able to transduce Vero and CRFK cells, and no GFP expression was observed with transduction of the negative control pseudotype (Table 5.2). Titres of pseudotypes remained similar to those previously produced using the VR1012 expression plasmid, therefore expressing RVSV glycoproteins using the T7 polymerase/promoter system did not appear to significantly improve the titre of retroviral pseudotypes.

Plasmid ratio	Cell line	RVFV	Δ Env	VSV
Number of GFP expressing cells				
1:1.5:1	BHK-21	8	0	>1000
	CRFK	90	0	>1000
	Vero	49	0	>1000
1:1:1	BHK-21	13	0	>1000
	CRFK	23	0	>1000
	Vero	16	0	>1000
1:1:4	BHK-21	0	0	>1000
	CRFK	0	0	>1000
	Vero	2	0	>1000

Table 5.2 Number of GFP expressing cells in cell lines transduced with Rift Valley fever virus pseudotypes produced using the vaccinia T7 polymerase/promoter expression system. MLV(RVFV) pseudotypes were produced in 293T cells using CMVi, CNCG and pTM1-GnGc in the presence of recombinant vaccinia virus as the source of T7 pol. Pseudotypes were harvested at 24 h and used to transduce BHK-21, CRFK and Vero cell lines. GFP expressing cells were counted manually by fluorescence microscopy at 48 h post transduction. The ratios represent the plasmid ratio of CMVi:CNCG:pTM1-GnGc

respectively. MLV(VSV) and MLV(Δ Env) were produced concurrently as positive and negative control pseudotypes.

5.2.2 Production of rhabdoviral pseudotypes bearing Rift Valley fever virus glycoproteins

Cifuentes-Monoz et al. (2010) successfully produced retroviral pseudotypes to ANDV, a hantavirus from the *Bunyaviridae* family, which were effectively neutralised by convalescent sera from ANDV-infected patients. In contrast to our study on retroviral RVFV pseudotypes, Cifuentes-Monoz demonstrated detectable ANDV glycoproteins in the supernatant of transfected cells. Pseudotype production using retroviral systems was attempted for hantaan and puumala hantaviruses and also SFTSV without success, however pseudotypes of these viruses were successfully produced to reasonable titres when a VSV pseudotype system was employed (Higa et al., 2012, Hofmann et al., 2013). Another hantavirus, Seoul virus, and the phlebovirus SFTSV have also been successfully pseudotyped using this system (Ogino et al., 2003, Tani, 2014, Hofmann et al., 2013). In 2014, Bukbuk et al. reported RVFV pseudotypes produced using the VSV pseudotype system, although no detail of the titres of such pseudotypes were discussed, they were successfully used to replace live virus in a RVFV pseudotype based neutralisation assay.

The VSV pseudotype system utilises a recombinant VSV in which the glycoprotein has been deleted (rVSV- Δ G). Due to the mechanism of VSV budding, which does not require glycoprotein to be present, this recombinant VSV can therefore be used to produce pseudotypes using the glycoprotein of heterologous viruses (Whitt, 2010), this method was first reported in 1997 for pseudotyping Ebola virus (Takada et al., 1997). We therefore questioned whether rVSV- Δ G could be used to generate RVFV pseudotypes in this study. Various reporter genes have been incorporated into the rVSV- Δ G virus and viruses expressing GFP (rVSV- Δ G*GFP), red fluorescent protein (RFP) (rVSV- Δ G*DsRed) and luciferase (rVSV- Δ G*luc) for this study were kindly provided by Dr. Michael Whitt (University of Tennessee). To determine optimal pseudotype production prior to pseudotyping

RVFV, the VSV system was used initially to produce rabies virus pseudotypes. Briefly, 293T cells were transfected with a rabies virus glycoprotein expression vector and incubated for 4 h at 37°C. Transfection medium was then replaced with low serum concentration media and incubated for a further 24 h. Transfected cells were infected with rVSV-ΔG at MOI 1 and adsorbed for 2 h, after which cells were extensively washed with serum free media. Fresh medium was added to the wells and further incubated at 37°C. Supernatants were harvested 24 h post infection, filtered and frozen at -80°C until required. This protocol differed slightly to the protocol suggested by Whitt et al. (2010), as 293T cells were used in place of BHK-21 cells, infection with rVSV-ΔG was carried out at a lower MOI than suggested and that rVSV-ΔG was removed after 2 hours where cells were additionally extensively washed. These alterations to the protocol were necessary, as prior to such modifications ΔEnv pseudotypes expressed significant levels of the reporter genes upon transduction of target cells, suggesting non-specific pseudotype production or carry over of reporter expressing virus. Protocols reported by Tani et al. (2011) recommend the removal of virus and extensive washing of cells for successful pseudotype production, we found this modification, in addition to a reduction in the MOI of rVSV-ΔG, removed the majority of non-specific pseudotype production. After optimising the VSV pseudotype system for rabies virus (VSV(rabies)), we produced VSV-derived RVFV pseudotypes using both pTM1-GnGc expression plasmid (with the addition of vaccinia virus) and RVF-GnGc. With all rVSV-ΔG constructs, expressing GFP, RFP and luciferase, VSV(RVFV) pseudotypes were not produced to a high titre, additionally positive cells were observed in target cells transduced with negative control ΔEnv pseudotypes (Figure 5.11). The positive control VSV(VSV) pseudotypes produced in parallel were always produced to a very high titre (Figure 5.11 and 5.12). In contrast to the MLV(rabies), in which high titres are achieved, the rabies virus pseudotypes produced using the VSV pseudotype system demonstrated only modest titres (Figure 5.12). Due to the poor pseudotype titres obtained using the VSV pseudotype system for RVFV pseudotypes, in addition to the non-specificity demonstrated upon transduction of the ΔEnv pseudotypes, the VSV(RVFV) pseudotypes were determined not to be suitable for use in a pNT.

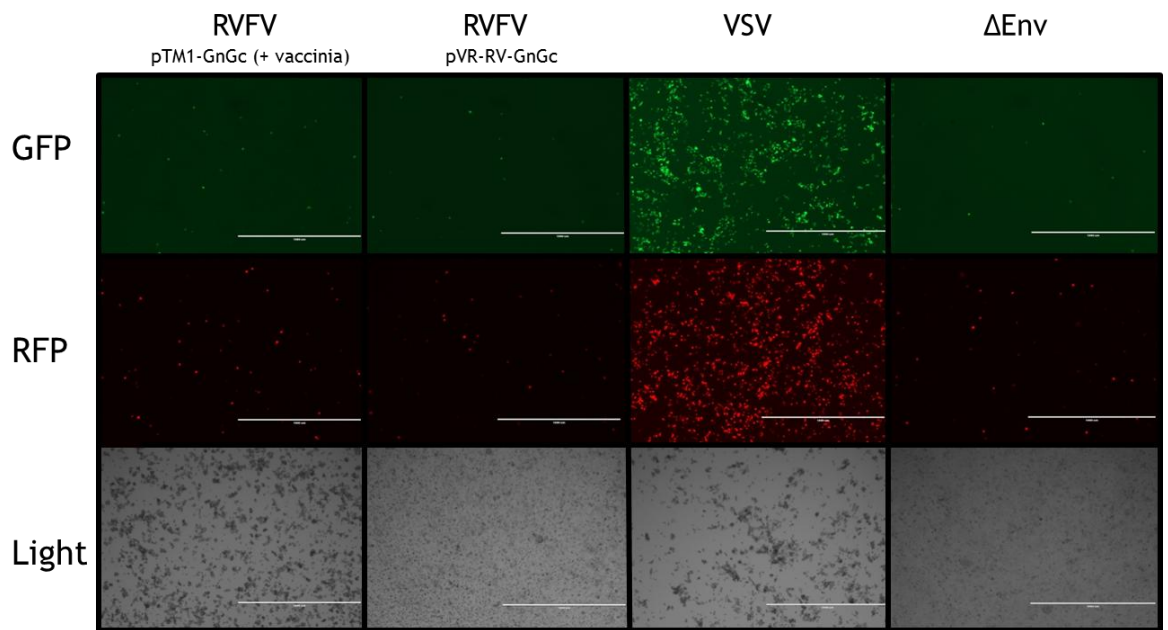


Figure 5.11 Reporter gene expression of Rift Valley fever virus pseudotypes produced using the rVSV-ΔG pseudotyping system. CRFK cells were transduced with pseudotypes bearing RVFV glycoproteins produced using rVSV-ΔG*DsRed and rVSV-ΔG*GFP. Cells were examined at 24 h post transduction. VSV and ΔEnv were produced in parallel and served as positive and negative control pseudotypes respectively.

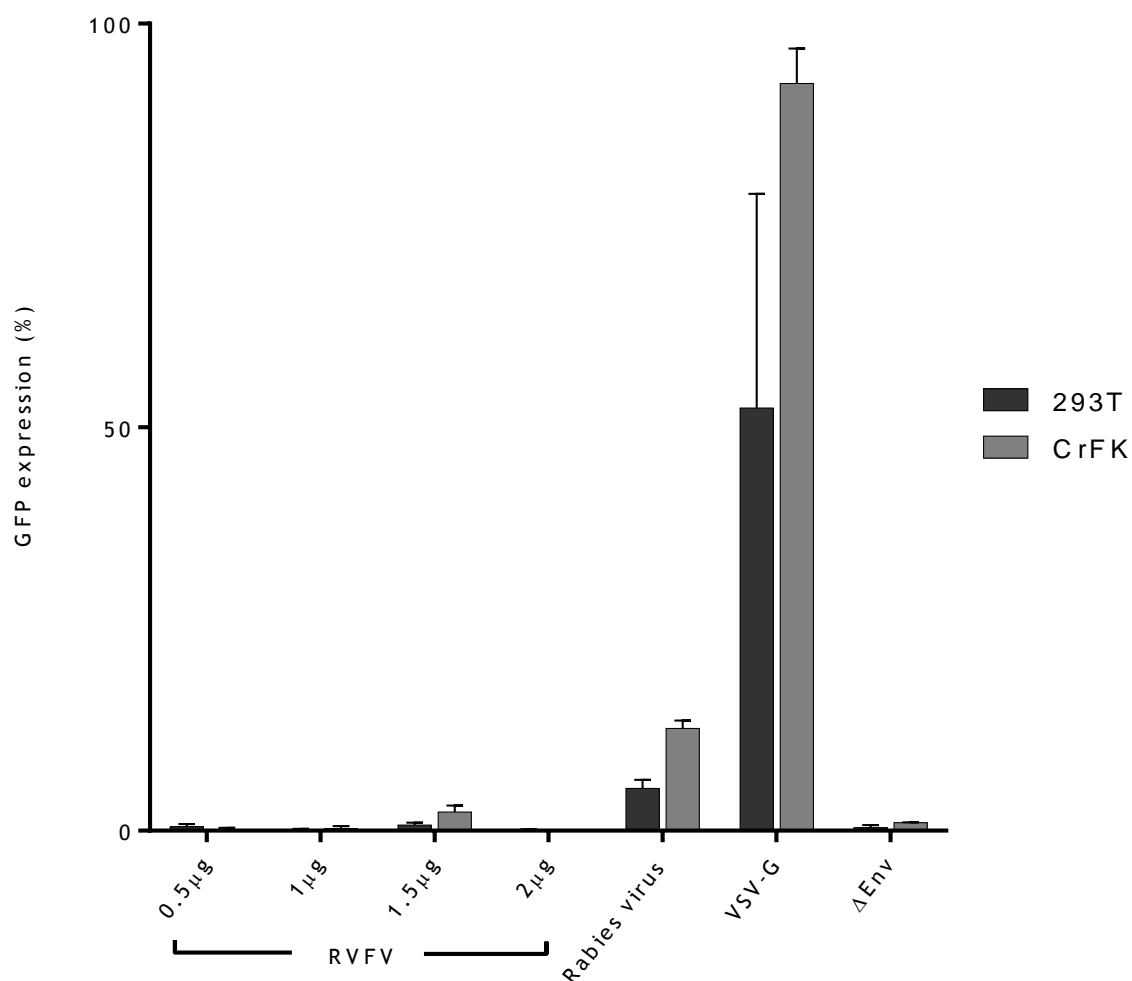


Figure 5.12 Expression of GFP following transduction with rVSV-ΔG pseudotypes. Pseudotypes were produced by transfecting RVF-GnGc into 293T cells followed by infection with rVSV-ΔG*GFP at 4 h. The resulting VSV(RVFV) pseudotypes were harvested at 24 h post infection. The concentrations represent the amount of RVF-GnGc plasmid transfected initially into 293T cells. 293T and CRFK cells were transduced with neat pseudotype and GFP expression was analysed by flow cytometry at 24 h post transduction. VSV(VSV-G) and VSV(rabies) pseudotypes were produced in parallel as positive controls and VSV(ΔEnv) was produced in parallel as a negative control.

5.2.3 Optimisation and validation of a Rift Valley fever virus neutralisation assay using luciferase-expressing Rift Valley fever virus

The low and inconsistent titres achieved when pseudotyping with RVFV glycoproteins suggested that an RVFV pNT would not be suitable for a reliable, high-throughput assay. We therefore designed an alternative neutralisation assay for the detection of RVF VNAb using a recombinant RVFV which incorporates a luciferase reporter gene. This recombinant virus, rMP12 Δ NSs:hRen, was kindly provided by Dr. Ben Brennan (University of Glasgow). The virus was derived from the MP-12 strain of RVFV in which the NSs coding sequence was replaced with a humanised *Renilla* luciferase. Upon infection and virus replication, luciferase expression can be measured in the infected cells using a luminometer. Initially, to determine the infectivity of the recombinant virus and to determine the optimum incubation time, BHK-21 cells were infected with rMP12 Δ NSs:hRen at an MOI of 1 and 5 and measured at 3, 6 and 24 h post-infection (hpi). Luciferase activity was detectable from 3 hpi with an approximate 22- and 117-fold increase in luciferase signal over background for cells infected with MOI of 1 and 5 respectively. At 6 hpi this increased to an approximate 2300- and 10000-fold increase. Luciferase levels were similar for both MOI 1 and 5 at 24 hpi, with an approximate 8000-fold increase over background (Figure 5.13). Based on the results of this experiment, we determined that for our RVFV luciferase neutralisation test (RVFV-luc NT) an intermediate MOI of 2 would be used and the assay would be analysed at 6 hpi, providing a clear distinction between virus infection and background signal and allowing a result to be reported 12 times faster than the current gold standard PRNT.

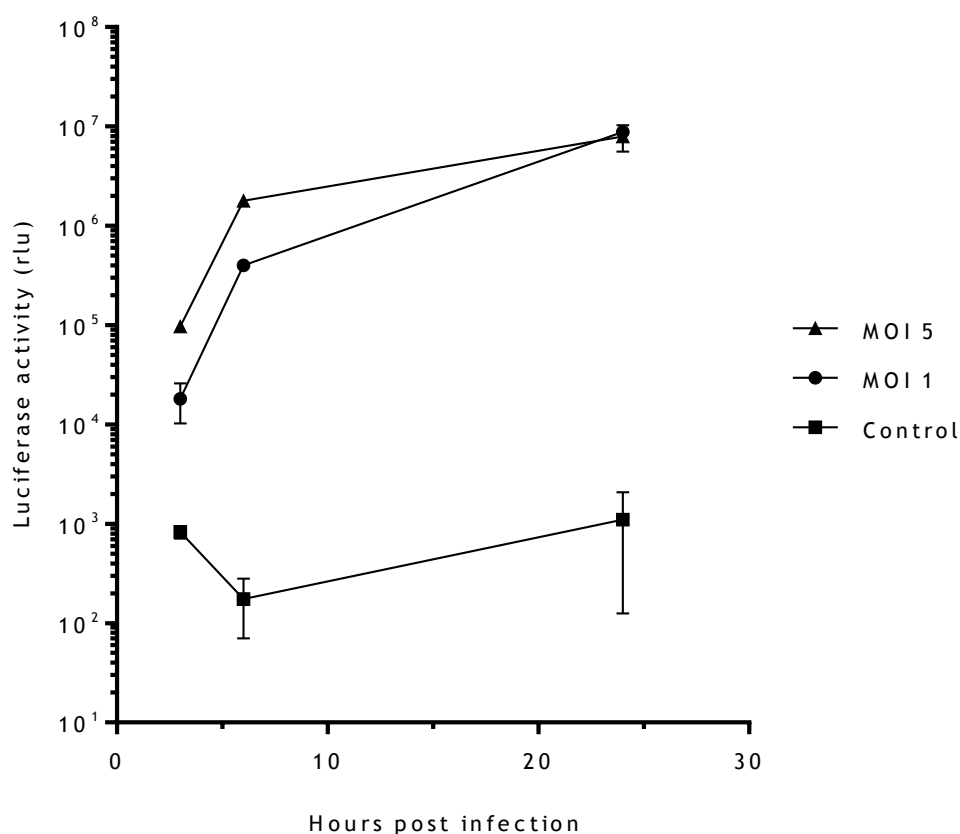


Figure 5.13 Luciferase expression following rMP12ΔNSs:hRen infection of BHK-21 cells. BHK-21 cells were infected with rMP12ΔNSs:hRen at an MOI of 1 and 5 while control cells were left uninfected to determine background luciferase signal. At the indicated time points, BHK-21 cells were lysed and luciferase signal measured in relative light units (rlu). Infections were performed in duplicate.

Seven serum samples which had previously been tested by a RVFV IgM capture ELISA were tested on the RVFV-luc NT to evaluate whether the assay could detect antibodies to RVFV. Three samples had tested negative by ELISA (112, 118 and 214) and four had tested positive by ELISA (35, 86, 2489 and 2543). A doubling dilution series, ranging from 1:10 to 1:1280, of these seven samples was tested by the RVFV-luc NT in duplicate. The percentage neutralisation was calculated using the following formula:

$$\text{Neutralisation (\%)} = \left[1 - \frac{(\text{mean sample rlu}) - (\text{mean uninfected cell rlu})}{\text{mean rMP12}\Delta\text{NSs:hRen infected cells rlu}} \right] \times 100$$

Neutralisation of rMP12 Δ NSs:hRen was observed in all RVFV antibody positive sera (Figure 5.14), with 75% (n=3) of the ELISA positive samples demonstrating 100% neutralisation in all dilutions up to 1:1280, with luciferase activity mimicking that of uninfected cells, suggesting complete neutralisation of rMP12 Δ NSs:hRen by sera. There appeared to be non-specific neutralisation in ELISA negative samples at all dilutions, with levels of luciferase expression not reaching the level in rMP12 Δ NSs:hRen infected cells in the absence of serum. To further evaluate the limit of detection of the assay, one ELISA positive (#86) and one ELISA negative (#118) sample were further diluted two-fold from 1:100 to 1:420000000 (Figure 5.15). The luciferase signal plateaued at serum dilutions of around 1:200 and 1:12800 in the negative and positive samples respectively. In both samples, the maximum luciferase signal reached was two-fold lower than untreated infected cells, this may be due to non-specific inhibition of the rMP12 Δ NSs:hRen by unknown factors in the serum.

To compare the RVFV-luc NT to the gold standard PRNT, a doubling dilution series of a mouse monoclonal antibody (4D4) known to neutralise RVFV was tested on both assays. The PRNT gave a PRNT₅₀ value of 1:128. For a relative comparison the luciferase expression of uninfected cells and luciferase expression at the maximum antibody dilution of 1:4096 were taken as 100% and 0% values respectively. Using these values a 50% reduction in luciferase expression was obtained at the 1:1024 antibody dilution, demonstrating an almost 10-fold increase in sensitivity of the RVFV-luc NT compared to the PRNT (Figure 5.16).

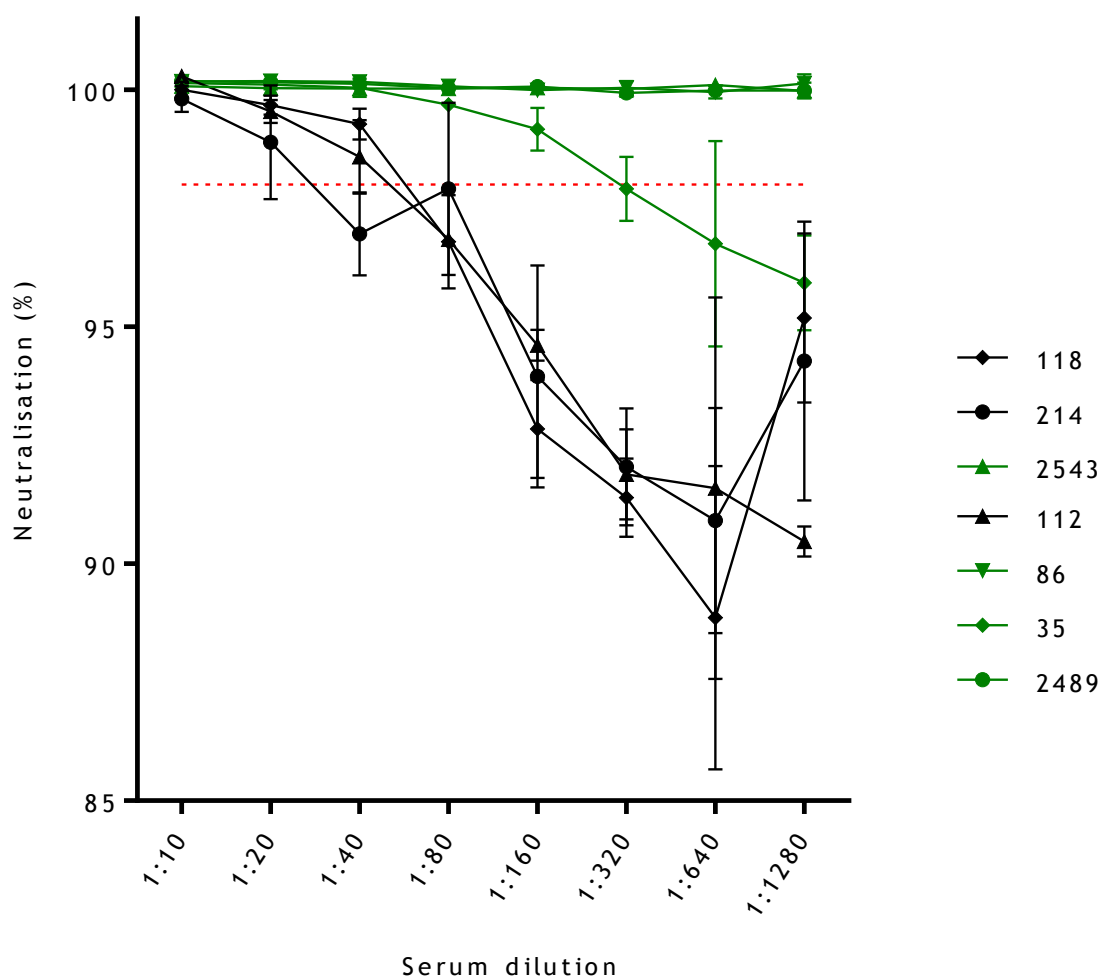


Figure 5.14 Evaluation of the RVFV-luc neutralisation test using samples of known Rift Valley fever virus antibody status. Seven buffalo sera which had previously been tested by RVFV IgM ELISA were serially diluted two-fold from 1:10 to 1:1280 and tested on the RVFV-luc NT, utilising rMP12ΔNSs:hRen at MOI 2 with luciferase activity measured at 6 hpi. Green lines represent samples testing positive by ELISA and those in black tested negative by ELISA for RVFV IgM. The red dashed line represents a level of 98% neutralisation.

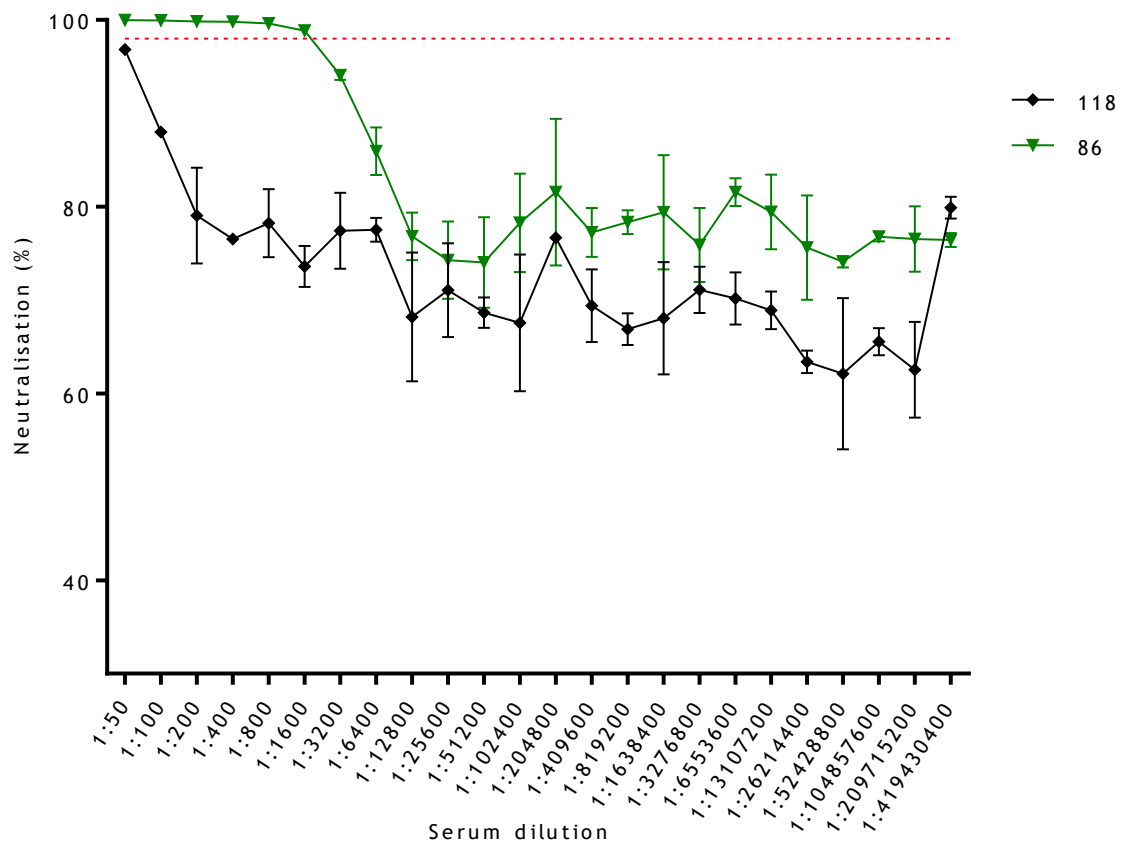


Figure 5.15 Extended dilution series of Rift Valley fever virus antibody positive and negative sera. The RVFV-luc NT was repeated on two of the samples previously tested in Figure 5.14, samples were further diluted from 1:50 to 1:419430400. Sample 118 had previously tested negative for RVFV IgM antibodies and sample 86 had tested positive. The red dashed line represents a level of 98% neutralisation.

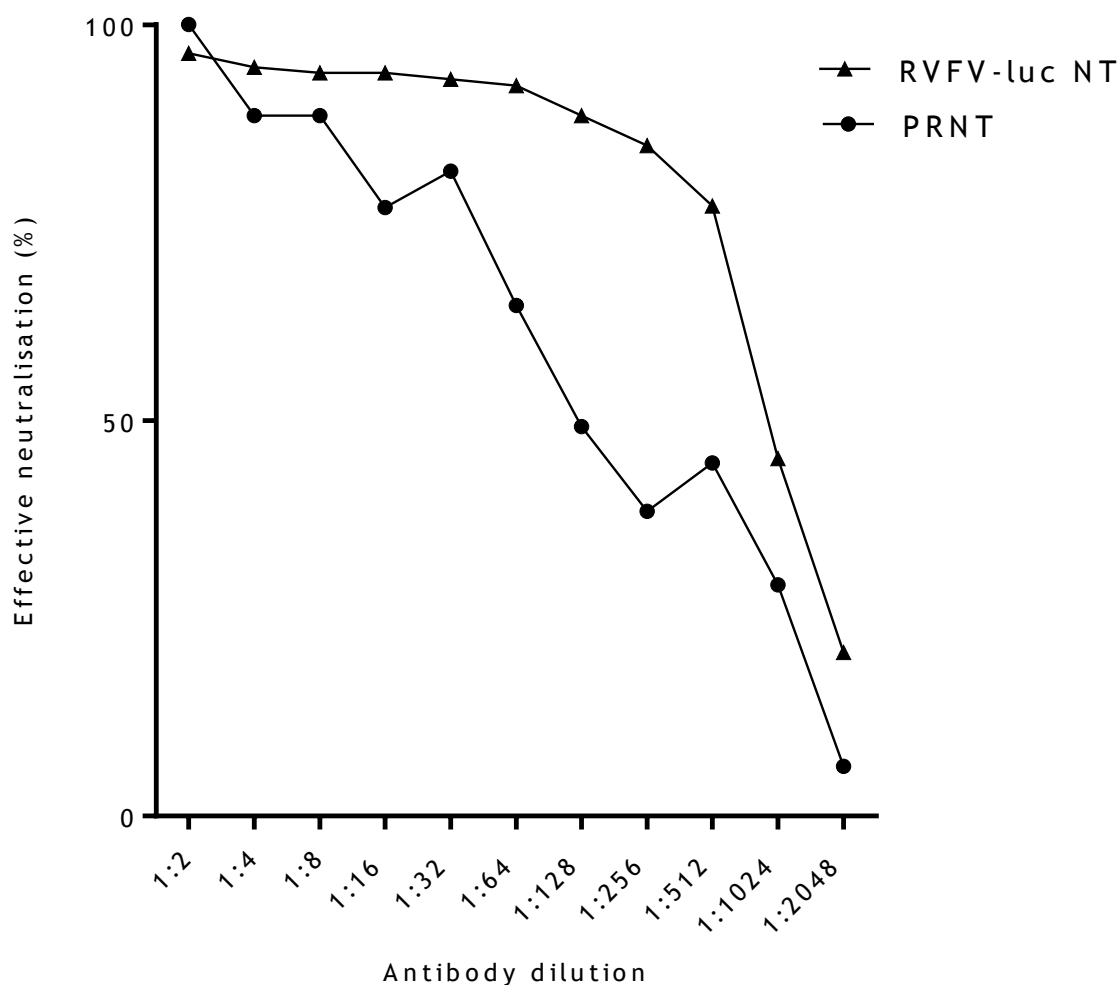


Figure 5.16 Comparison of a dilution series of a neutralising antibody to Rift Valley fever virus when measured by PRNT and RVFV-luc NT. A doubling dilution series of the mouse monoclonal RVFV neutralising antibody (4D4) was tested on both the PRNT and RVFV-luc NT. The PRNT was performed using the MP-12 strain of RVFV and plaque reduction was analysed at 72 hpi. Luciferase expression in the RVFV-luc NT was analysed at 6 hpi. The PRNT was performed by Dr. Felix Kreher and the RVFV-luc NT was performed by Dr. Stephen Welch.

Ideally the RVFV-luc NT would be performed using a single serum dilution to allow for high-throughput serum analysis, which is preferable for large scale serosurveillance. The data presented in Figure 5.14 suggest that using a single serum dilution of 1:100 and a cut-off of $\geq 98\%$ neutralisation would successfully differentiate between RVF VNAb positive and negative sera. To validate the RVFV-luc NT using a serum dilution of 1:100 and a cut-off for neutralising activity of $\geq 98\%$, 53 serum samples of known RVFV antibody status were tested using the RVFV-luc NT. These samples comprised 19 lion serum samples previously tested by the gold standard PRNT, kindly provided by Prof. Craig Packer (University of Minnesota). Five of the samples were positive for RVF VNAb with PRNT₅₀ titres of 160, two samples were considered inconclusive with a titre of 40, while 12 samples were negative with PRNT₅₀ titres of ≤ 10 . A further 34 samples (21 African buffalo samples and 13 serum samples from Thomson's gazelle) which had previously been tested by RVFV IgM ELISA, were also utilised in the validation panel. One Thomson's gazelle sample and nine buffalo samples were positive for RVFV IgM, two buffalo samples were equivocal by ELISA and the remaining samples were negative. Samples were tested at a dilution of 1:100 in duplicate and a level of neutralisation was calculated using the aforementioned formula, a level of $\geq 98\%$ was considered positive for the presence of RVF VNAb. We demonstrated a 100% correlation of results obtained using the RVFV-luc NT with the lion samples tested by PRNT, with all negative and positive samples being correctly interpreted, the two inconclusive samples were negative by RVFV-luc NT (Figure 5.17a). When comparing the results of the RVFV-luc NT to the RVFV IgM ELISA, 100% (21/21) of the negative samples were correctly interpreted and 91% (10/11) of the positive samples were correctly interpreted, one of the inconclusive samples tested positive and one negative by RVFV-luc NT (Figure 5.17b). Using these results (excluding inconclusive samples) the sensitivity and specificity of the RVFV-luc NT are 94% (95% CI 69.8-99.8%) and 100% (95% CI 89.4-100%) respectively.

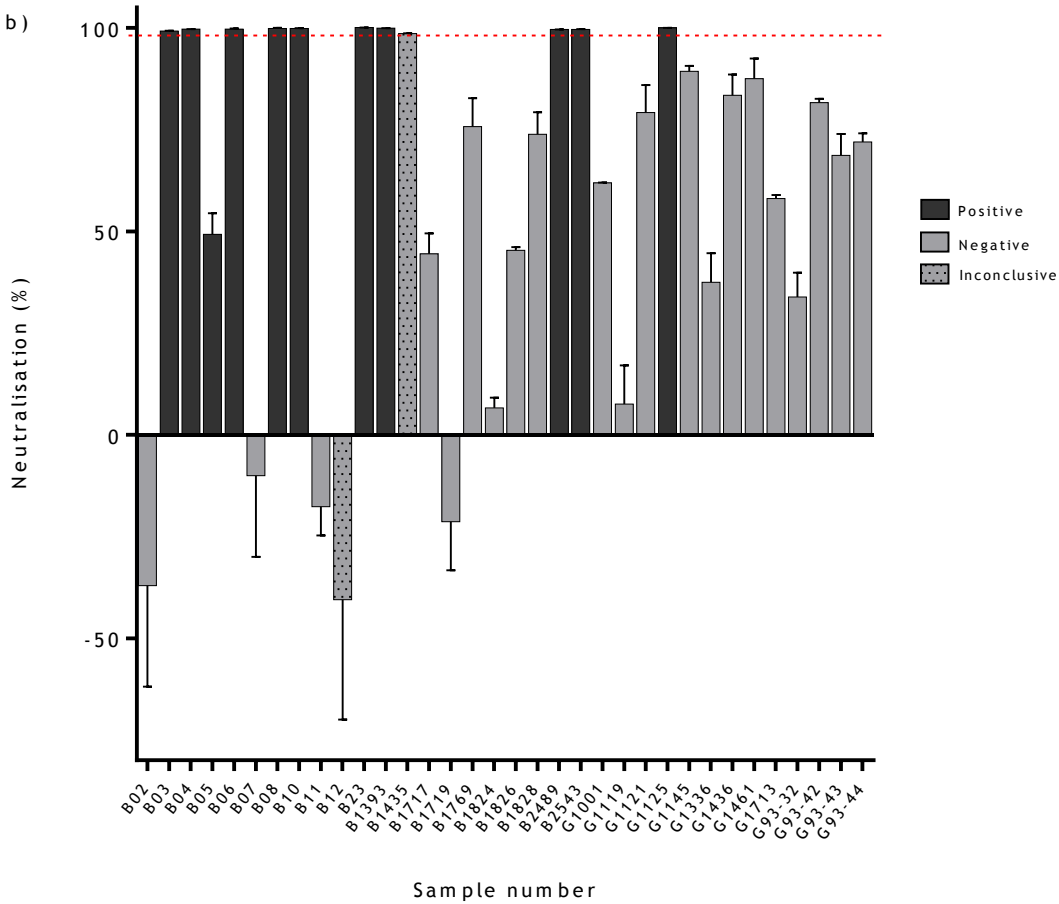
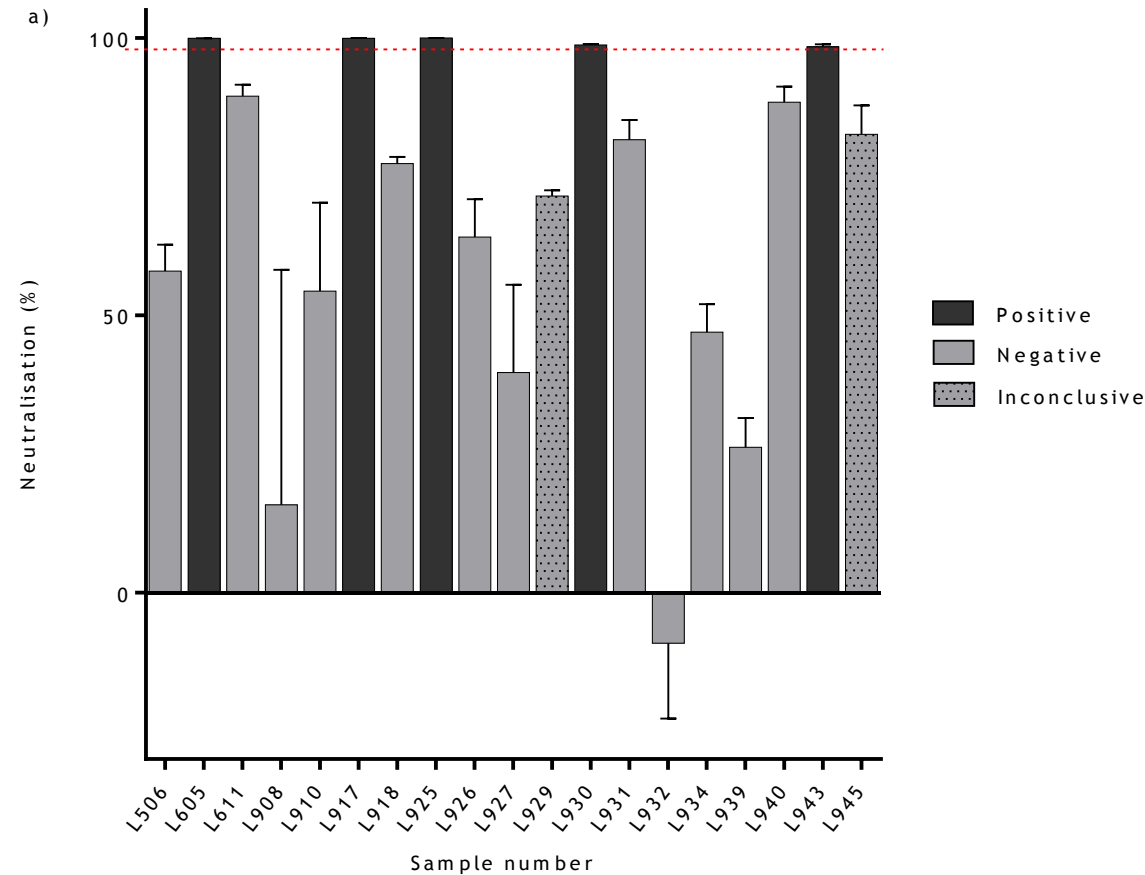


Figure 5.17 Validation of the RVFV-luc neutralisation test using 53 samples of known RVFV antibody status. Fifty-three samples previously tested for antibodies to RVFV were tested by RVFV-luc NT, in duplicate using a single serum dilution of 1:100 and a cut-off of $\geq 98\%$. a) Nineteen lion serum samples were tested on the RVFV-luc NT which had previously been tested by RVFV PRNT, a neutralising titre of <40 was considered negative in the PRNT, a titre of 40 was inconclusive and >40 was considered positive. b) Thirty four samples were tested on the RVFV-luc NT which had previously been tested by RVFV IgM capture ELISA. The colours of the bars represent the RVFV antibody status at previous testing. The red dashed line represents the cut-off of 98% neutralisation.

To determine the optimal percent neutralisation threshold for the RVFV-luc NT, a receiver operating characteristic (ROC) curve was performed using the results of the validation panel ($n=49$) (Figure 5.18). The RVFV-luc NT demonstrates excellent discrimination between RVF VNAb positive and negative samples, with an area under curve (AUC) of 0.97 (95% CI 0.90-1.03) and $p<0.0001$. The optimal percent neutralisation cut-off calculated by the ROC analysis was 93.9%, which would provide an assay sensitivity of 93.8% (95% CI 69.8-99.8%) and specificity of 100% (95% CI 89.4-100%). However, Figure 5.14 demonstrates that sera negative for RVF VNAb, at dilutions of 1:160, demonstrate percent neutralisation of around this cut-off (92.8%, 94.0% and 94.6%), therefore we decided to retain the more stringent cut-off of $\geq 98\%$ neutralisation as evidence of RVF VNAb. Although this more stringent cut-off may reduce the sensitivity of the assay slightly, it was deemed that the specificity of the assay was of greater significance as the aim of this project was to provide reliable serosurveillance data.

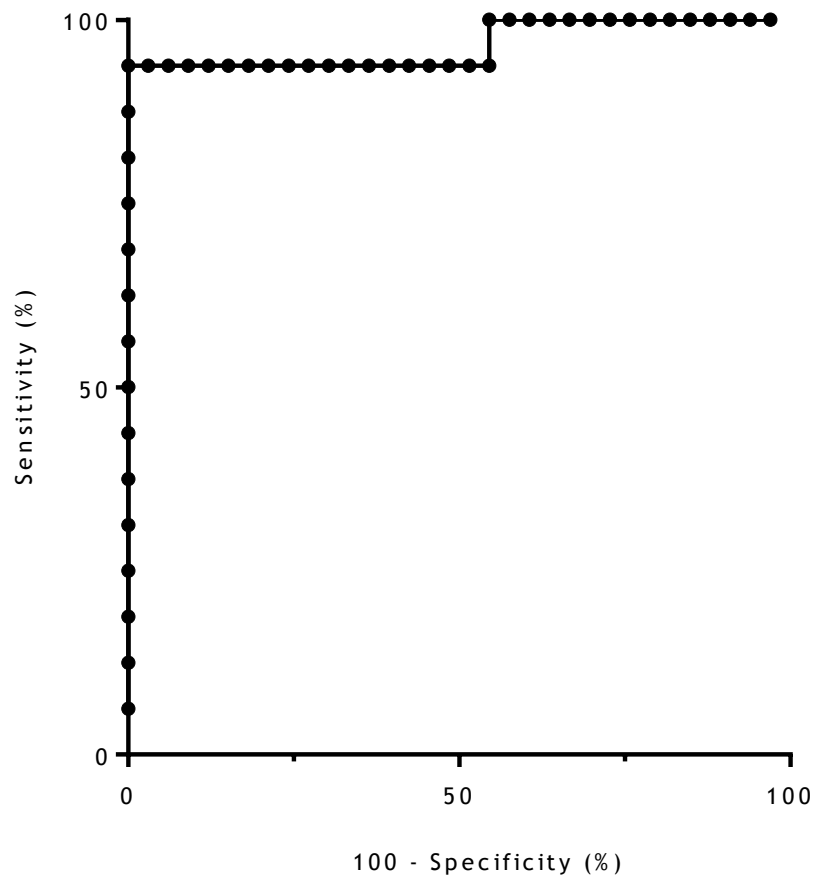


Figure 5.18 Receiver operator characteristic (ROC) analysis of RVFV-luc neutralisation test. ROC curve analysis of sensitivity and 100-specificity (%) data for the RVFV-luc NT based on 49 samples of known RVFV antibody status, which had previously been tested using the PRNT or RVFV IgM capture ELISA. AUC=0.966.

To investigate inter-assay variation in the RVFV-luc NT 16 samples were selected from five different species (lion n=2, gazelle n=5, buffalo n=4, cattle n=3, goat n=2) and tested on two different days, using different BHK-21 cells and different aliquots of rMP12 Δ NSs:hRen. The results demonstrated a good agreement between both assays with 88% (14/16) of samples being classified as either RVF VNAb positive or negative on both tests (Figure 5.19). The results of two samples were discrepant between the tests. One buffalo sample tested negative on the first test but positive on repeat testing, one cattle sample tested negative on the first test and positive on repeat testing, however in both samples the negative result fell close to the cut-off value of $\geq 98\%$ (97.11% and 94.63%). This data supports the use of the lower cut-off of $>94\%$ in the RVFV-luc NT, reinforcing the need for further validation of the assay, and the potential cut-off, by comparing field samples tested by RVFV-luc NT and the gold standard PRNT.

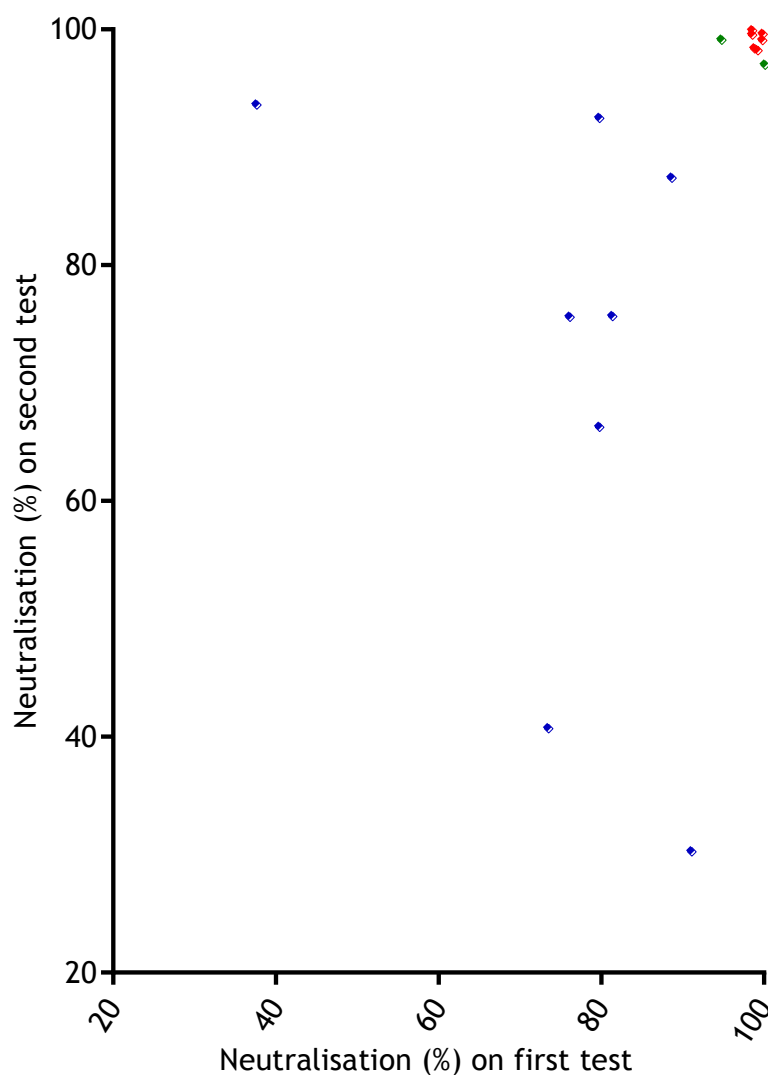


Figure 5.19 Inter-assay variation in the RVFV-luc NT was investigated by testing samples on two different days. Sixteen samples from five different species were tested on the RVFV-luc NT on two different days using different aliquots of virus and a different batch of BHK-21 cells to assess the level of inter-assay variation. Red diamonds represent samples that tested RVF VNAb positive on both tests (n=6), blue diamonds represent samples testing RVF VNAb negative on both tests (n=8) and green diamonds represent samples testing RVF VNAb positive on one test and negative on repeat testing (n=2).

In the rabies virus pNT, we demonstrated that non-specific neutralisation in serum samples could cause false positive results. This problem was addressed by testing serum against an unrelated pseudotype to detect non-specific neutralisation, samples that demonstrated non-specific neutralisation were investigated further. There is no non-specific neutralisation control included in the RVFV-luc NT. To determine if non-specific neutralisation may be a factor in the RVFV-luc NT, we tested a random selection of serum samples on the RVFV-luc NT and further tested them using an identical method but replacing the rMP12 Δ NSs:hRen with rBUN Δ NSs:hRen. The rBUN Δ NSs:hRen virus is a recombinant bunyamwera virus expressing *Renilla* luciferase kindly provided by Dr. Xiaohong Shi (University of Glasgow). Bunyamwera virus is a member of the *Orthobunyavirus* genus of the *Bunyaviridae* family, which is present throughout the world (Tauro et al., 2009). To determine if non-specificity affected the classification of neutralising or non-neutralising serum, we tested 73 samples using both the RVFV-luc NT and the BUNV-luc NT, these samples comprised of samples from lions (n=10), buffalo (n=14), gazelle (n=11), cattle (n=14), sheep (n=10) and goats (n=14). Twenty-five of these samples tested positive for RVF VNAb (lions n=3, buffalo n=5, gazelle n=3, cattle n=6, sheep n=2 and goat n=6), the remaining 48 tested negative by RVFV-luc NT. Eleven samples neutralised rBUN Δ NSs:hRen at a level of $\geq 98\%$ (lions n=3, buffalo n=4, gazelle n=1, cattle n=1, sheep n=1 and goat n=1), the remaining samples were non-neutralising (Table 5.3). All of the buffalo samples testing positive on the BUNV-luc NT were also RVF VNAb positive, the remaining seven samples demonstrating neutralisation on the BUNV-luc NT were negative when tested by RVFV-luc NT. The proportion of bunyamwera VNAb positive samples amongst RVF VNAb positive and negative samples were 16% and 15% respectively, therefore non-specific neutralisation did not appear to be an issue in these samples. Antibodies to the bunyamwera serogroup have been reported from a variety of both domestic and wildlife species (Issel et al., 1970, Campbell et al., 1989, Fagbami and Fabiyi, 1975, Sahu et al., 2002) and therefore the presence of antibodies to bunyamwera virus in sera tested in this study is not entirely unexpected.

		Bunyamwera VNAb		
Rift Valley fever VNAb		Positive	Negative	Total
	Positive	4	21	25
	Negative	7	41	48
	Total	11	62	73

Table 5.3 Rift Valley fever and bunyamwera virus neutralisation by serum samples. Seventy-three serum samples from five species were tested using the RVFV-luc NT and BUNV-luc NT for neutralising antibodies to RVFV and BUNV respectively. Tested sera comprised of lion (n=10), buffalo (n=14), gazelle (n=11), cattle (n=14), sheep (n=10) and goat (n=14) samples, tested at a single serum dilution of 1:100. A level of $\geq 98\%$ was considered evidence of VNAb in these assays.

5.3 Discussion

The current gold standard serological assay for evidence of RVFV infection is the VNT, which is highly specific and is the World Organisation for Animal Health (OIE) prescribed test for international trade (OIE, 2012a). However, the PRNT is laborious, demands technical expertise, requires 5-7 days for completion and most significantly, as live virus is required, the PRNT must be performed under BSL-3 conditions. Due to the biosafety implications and the incubation time required for the VNT it has limited use for large scale serosurveillance. The ELISA is the most commonly utilised method of RVFV antibody detection for sero-epidemiology (Mansfield et al., 2015) and commercial ELISAs based on the recombinant RVFV nucleoprotein are available. However, our laboratory and others (Faburay et al., 2013, Niklasson et al., 1984) have encountered a high degree of non-specific background when using RVFV ELISAs, which causes difficulty in the interpretation of results. Additionally, the ELISA is species-

specific and requires revalidation when sera from different species are tested. Currently the majority of commercial ELISAs are validated for humans or for domestic and wild ruminants only (Paweska et al., 2005), however a multi-species ELISA has been developed more recently. The reservoir species of RVFV remain unknown, with potential reservoir species ranging from rats to bats (Olive et al., 2012), hence pan-species serological assays for RVFV surveillance are required. Studies have also demonstrated cross-reactivity of the nucleoproteins of members of the *Phlebovirus* genus (Wu et al., 2014, Xu et al., 2007, Tesh et al., 1982, Szymczak et al., 2015), which renders these commercial ELISAs inadequate for serosurveillance in areas where other phleboviruses circulate, or indeed where as yet unknown phleboviruses may be circulating. The VNT is considered the most specific serological assay for RVFV (Beechler et al., 2015), as there is little or no cross-reactivity of phleboviral VNABs (Pepin et al., 2010), in addition the assay is not affected by the species being tested. Previous studies comparing RVFV serology using both ELISA and VNT have demonstrated high proportions of discrepant results, with the ELISA reporting high numbers of positives which cannot be confirmed by VNT (Evans et al., 2008, Gray et al., 2015), one study reported seroprevalence of RVFV in sheep and goats in Kenya as 17% when tested by ELISA but only 4% when the same samples were tested by VNT (Kariithi et al., 2010). Therefore, highly specific RVFV sero-diagnostic assays are required for generating reliable epidemiological evidence of RVFV circulation and maintenance, such assays require the specificity of the VNT but the ease, high-throughput and low biosafety requirements associated with an ELISA.

In this study initial attempts at developing such an assay focussed on the development of a pNT, following on from the success of the rabies virus pNT developed in Chapter 3. However, in this study MLV(RVFV) pseudotypes were produced at low titres, and although successfully neutralised by RVFV specific VNAB, these low and inconsistent titres made the RVFV pNT unfeasible for high-throughput serological screening. Successful incorporation of viral glycoproteins into pseudotype particles is dependent on the intrinsic cell localisation of the glycoprotein (Whitt, 2010, Sandrin and Cosset, 2006). Little or no glycoprotein expression has been observed on the plasma membrane of cells infected with viruses of the *Bunyaviridae* family (Pettersson and Melin, 1996). The glycoproteins of RVFV accumulate in the Golgi apparatus due to a Golgi

retention motif situated in the cytoplasmic tail of Gn (Gerrard and Nichol, 2002) and as with all bunyaviruses RVFV buds from the Golgi (Andersson et al., 1997). It can be assumed that during transfection GnGc proteins are also expressed on the Golgi membrane, for successful pseudotype production glycoprotein expression is required at the plasma membrane where retroviral and rhabdoviral pseudotypes bud. The small number of RVFV pseudotypes produced in our experiments could be due to a limited number of pseudotype particles which assemble and bud from endosomal vesicles, which have previously been reported (Sandrin and Cosset, 2006, Tani, 2014). In our study, attempts to improve glycoprotein expression using a T7 polymerase/promoter system did not sufficiently improve RVFV pseudotype titres. Filone et al. (2006) achieved significantly high cell surface expression of RVFV glycoproteins when the M segment of RVFV was cloned into a Venezuelan equine encephalitis virus vector, which was further utilised to develop a cell-cell fusion assay. If future attempts were made to pseudotype RVFV, such alphavirus replicon particles may be considered to achieve sufficient plasma membrane expression of RVFV glycoproteins and in turn allow for high titre RVFV pseudotypes to be produced. Previous studies have also reported glycoproteins previously unsuccessfully incorporated into pseudotypes being successfully incorporated when the cytoplasmic tail of the glycoprotein was deleted or replaced with the cytoplasmic tail of another virus. Sandrin et al. (2006) reported unsuccessful pseudotypes produced when the wild type glycoprotein of the cat endogenous retrovirus RD114 was transfected with retroviral vectors, however pseudotypes were successfully produced when the cytoplasmic tail of RD114 was replaced with the cytoplasmic tail of MLV. Further studies have also reported successful incorporation of viral glycoproteins when chimeric MLV cytoplasmic tails were produced (Lindemann et al., 1997, Christodoulopoulos and Cannon, 2001) and other studies have used similar methodologies using chimeric VSV cytoplasmic tails (Carpentier et al., 2012). Additionally, measles lentiviral pseudotypes have been described in which wild type measles glycoproteins F and H were only successfully incorporated into pseudotype particles when their cytoplasmic tails were truncated (Frecha et al., 2008). As the retention motif of the RVFV glycoproteins is in the cytoplasmic tail, such strategies could also be attempted to increase RVFV glycoprotein expression at the plasma membrane and hence increase RVFV pseudotype titres.

Many viruses have been pseudotyped using the VSV pseudotyping system, including influenza C, Ebola, measles, hepatitis C virus and hantaviruses (Hanika et al., 2005, Takada et al., 1997, Tatsuo et al., 2000, Tamura et al., 2005, Ogino et al., 2003), these include hantaviruses that could not be pseudotyped using retroviral vectors (Higa et al., 2012). To our knowledge the only phleboviruses that have been successfully pseudotyped are SFTSV and RVFV, in both instances pseudotypes were produced using the VSV pseudotype system. However, these phlebovirus pseudotypes were produced at low titres, around 100-fold lower than the control pseudotypes produced using the glycoprotein of VSV (Tani, 2014). We used the VSV pseudotype system to produce VSV(RVFV) pseudotypes and although reporter expression was noted on transduction of target cells, negative control pseudotypes lacking glycoprotein also produced positive cells upon transduction. VSV has been shown to bud at a low level in the absence of any glycoprotein (Robison and Whitt, 2000) and non-specific cellular uptake of envelope deficient retroviral pseudotypes has been reported previously (Voelkel et al., 2012), a combination of these two events could result in non-specific reporter expression seen upon transduction of VSV(Δ Env) pseudotypes. However, we noted increased expression of reporter genes upon transduction when cells were not extensively washed after removal of rVSV- Δ G, suggesting reporter expression could be due to residual carry over of the rVSV- Δ G. Previous studies reporting use of the VSV pseudotyping system have incubated transfections with polyclonal anti-VSV serum to neutralise residual rVSV- Δ G, hence such non-specific carry over seems to have been a factor in other studies (Hofmann et al., 2013, Hanika et al., 2005). When pseudotypes are produced to high titres such non-specific background may be negligible, however with such low VSV(RVFV) pseudotype titres achieved, and background expression levels not significantly different, such a background is unacceptable. Pseudotypes are a powerful tool for studying cell entry, gene transfer, vaccine candidates and for measuring neutralising antibodies, and although many viruses have been successfully pseudotyped some viruses may require further modifications before pseudotypes can be produced to a useable titre.

With the RVFV pNT not a viable option for screening for VNAb, we investigated alternative methods for RVF VNAb detection. The RVFV-luc NT developed in this Chapter is a novel assay for detecting RVF VNAb, which allows for high-

throughput, rapid and sensitive screening of serum regardless of host species. In comparison to the PRNT which can take up to 7 days to perform, and indeed the pNT which requires 48 h incubation, the RVFV-luc NT is incubated for only 6 h, resulting in a huge reduction in turnaround time. An additional improvement of this assay over the PRNT is that results are reported in absolute number and therefore it does not rely on the subjective nature inherent in interpreting viral plaque formation, hence inter-assay variability is greatly reduced. Comparison of 50% neutralisation values of a monoclonal neutralising antibody measured on both the PRNT and RVFV-luc NT demonstrated increased sensitivity of the RVFV-luc NT assay over PRNT, however this result should be additionally confirmed using a panel of RVF VNAb positive sera. The RVFV-luc NT also lends itself to full automation, as with appropriate robotic and liquid handling systems the entire process could be automated once the cells have been prepared, offering huge advantages when large scale serosurveys are required. In this study the RVFV-luc NT was performed at BSL-3 as the MP-12 strain of RVFV is a live attenuated vaccine strain, however in the United States only BSL-2 containment is required for virus manipulation (CDC, 2009). Considering the recombinant virus used in the RVFV-luc NT is an attenuated strain, and the nonstructural (NSs) gene has been deleted, BSL-3 requirements for this strain could potentially be downgraded in the United Kingdom, in which case the RVFV-luc NT will be further improved by lowering the biosafety requirements of the assay. We have demonstrated serum cytotoxicity in the rabies virus pNT, which may cause false positive results. As the RVFV-luc NT is analysed using a luminometer, cell cytotoxicity cannot be ascertained through microscopy as it would be in a conventional VNT or pNT. However, Garcia et al. (2014) have described a high-throughput luciferase assay in which a metabolic viability dye can be incorporated into the assay to attain an assessment of cell cytotoxicity using the luminometer. Such methodology should be considered to improve the reliability of the RVFV-luc NT, if this assay were to be further developed, ensuring cell viability in the assay would be an important improvement.

A limitation of this study was the small number of serum samples used for validation, 53 previously analysed samples were tested on the RVFV-luc NT, of which only 19 were tested by the gold standard PRNT. Ideally for a more reliable calculation of sensitivity and specificity, a larger sample size should be tested by

the gold standard for comparison with RVFV-luc NT, unfortunately due to time constraints this was not possible during this project. To establish a high-throughput assay a single serum dilution was used rather than titration to endpoint. However, ideally for further validation of the assay serum samples should be further titrated to give a quantification of RVF VNAb in the sample. Again due to time constraints in the study this was not done. To improve the assay, during serosurveillance studies serum samples testing positive on the RVFV-luc NT should be further titrated to allow an end point titre to be calculated. With this approach the stringent cut-off of 98% neutralisation selected in this study could be lowered, as high sensitivity is preferable to high specificity in a screening assay, as false positives will be detected upon full serum titration.

Understanding the dynamics of RVFV during both epidemics and inter-epidemic periods relies on robust serosurveillance data from a wide range of host species. The aim of this research was to develop a high-throughput, reliable VNT for use with multiple species, allowing collection of robust serosurveillance data and in turn improving the understanding of RVFV transmission, reservoir species and host susceptibilities. The RVFV-luc NT is employed in Chapter 6 to investigate some of these questions.

Chapter 6. A sero-epidemiological study of Rift Valley fever virus in Tanzania

6.1 Introduction

Rift Valley fever virus (RVFV) is an arbovirus spread by mosquito vectors which can cause severe disease in both humans and animals. The virus is considered endemic in sub-Saharan Africa (Mansfield et al., 2015), and prior to the year 2000 had not been detected outside the African continent, but has since spread to the Arabian Peninsula (Balkhy and Memish, 2003) and the Comoros Archipelago (Sissoko et al., 2009). Outbreaks of RVFV occur after periods of heavy rainfall and flooding, which provide optimum breeding conditions for mosquito vectors. Transovarian transmission of RVFV has been observed in mosquitoes (Linthicum et al., 1985) and it is hypothesised that infected, diapause eggs may survive in shallow wetland areas during inter-epidemic periods (IEP). After extensive flooding large numbers of these infected eggs hatch and an ensuing RVFV epidemic occurs, resulting in extensive transmission to animals and subsequent amplification of the virus.

Rift Valley fever is mainly considered a disease of domestic ruminants and humans. However, many other animals have exhibited symptomatic disease associated with RVFV infection, including camels (El Mamy et al., 2011), African buffalo and waterbuck (Beechler et al., 2015), while many more apparently healthy animals from diverse species have been found to be seropositive for RVFV-specific antibodies. Antibodies to RVFV have been reported in many wild animals including African buffalo (LaBeaud et al., 2011), impala (Capobianco Dondona et al., 2016) elephants (Evans et al., 2008), giraffes (Bird et al., 2008), black rhino (Lwande et al., 2015) and in one study RVFV antibodies were detected in lions (House et al., 1996). It is widely hypothesised that RVFV does

not circulate during IEP and transmission occurs only during outbreaks, however antibodies to RVFV have been detected both in animals, and in humans, who have only lived through IEP (Sumaye et al., 2013, LaBeaud et al., 2008, Gray et al., 2015). Much remains to be understood regarding the mechanisms of inter-epidemic persistence and circulation in mammalian host species.

The gold standard serological assay for detecting antibodies to RVFV is the virus neutralisation test (VNT), however this test is both time consuming, technically demanding and requires high containment facilities, and as a result, can be performed in very few laboratories in RVFV endemic countries in Africa. ELISAs are widely utilised for sero-epidemiological studies of RVFV, however results of ELISAs have been shown to correlate poorly with results from the VNT (Gray et al., 2015, Evans et al., 2008, Kariithi et al., 2010, Boussini et al., 2014). Robust serological evidence is imperative to understanding the complex, multi-host ecology of RVFV. In Chapter 5 we described a neutralisation assay in which wildtype virus is replaced with a recombinant RVFV expressing luciferase. This assay (RVFV-luc NT) allows for high-throughput testing using a single serum dilution of 1:100 and unlike many RVFV ELISAs, allows testing of serum from any species. We established a cut-off value of $\geq 98\%$ effective neutralisation as evidence of RVF virus neutralising antibodies (VNAb) and this cut-off was utilised in these studies. In this Chapter we describe the use of the RVFV-luc NT to investigate the presence of VNAb to RVFV in the unique archive of wildlife and livestock sera, collected over the past 20 years, from the Serengeti ecosystem. Using linked epidemiological data we aim to provide preliminary data to enable us to further understand the epidemiology of RVFV in Tanzania.

The study area comprises the Serengeti National Park (SNP), Ngorongoro Conservation Area (NCA) and the surrounding areas in Northern Tanzania (Figure 6.1). The SNP encompasses diverse wildlife communities and is a protected area which is bordered to the North by the Maasai Mara National Reserve, Kenya. The NCA is a wildlife protected area, however also functions as a multiple land use area, with wildlife, livestock and humans co-existing. There is a large pastoral Maasai population which live and herd cattle in the NCA. The area to the North West of the SNP is the Serengeti district which is inhabited by agro-pastoralist communities. To the East of the SNP and North of the NCA is Loliondo district which is inhabited by pastoralist communities. There are no physical boundaries

separating wildlife protected areas and human settlements (Lembo et al., 2008), hence wildlife and livestock are potentially in close contact.

Rift Valley fever virus was first documented in Tanzania in 1930 and since then periodic outbreaks of the disease have occurred in the country, major epidemics were reported in 1947, 1957, 1977/1978, 1997/1998 and 2006/2007. The largest RVFV outbreak in Tanzania was the most recent outbreak which affected 175 villages in North East and Central Tanzania in 2006/2007 (Sindato et al., 2014). A total of 144 human deaths were reported from this outbreak (Mohamed et al., 2010) and there were 32,000 reported cases in animals (Nanyingi et al., 2015). In addition to human fatalities, RVFV outbreaks also have significant socio-economic implications, to the extent that the 2006/2007 RVFV outbreak in Tanzania was declared a national disaster (Fyumagwa et al., 2011).

In this Chapter we perform three sero-epidemiological studies of RVFV in Northern Tanzania. The first study explores RVFV seroprevalence in lions living in the SNP; the second study investigates two wild ruminant species (African buffalo and gazelle) for evidence of RVF VNAb; and the third study is a longitudinal serosurvey of livestock from the Serengeti and Loliondo districts.

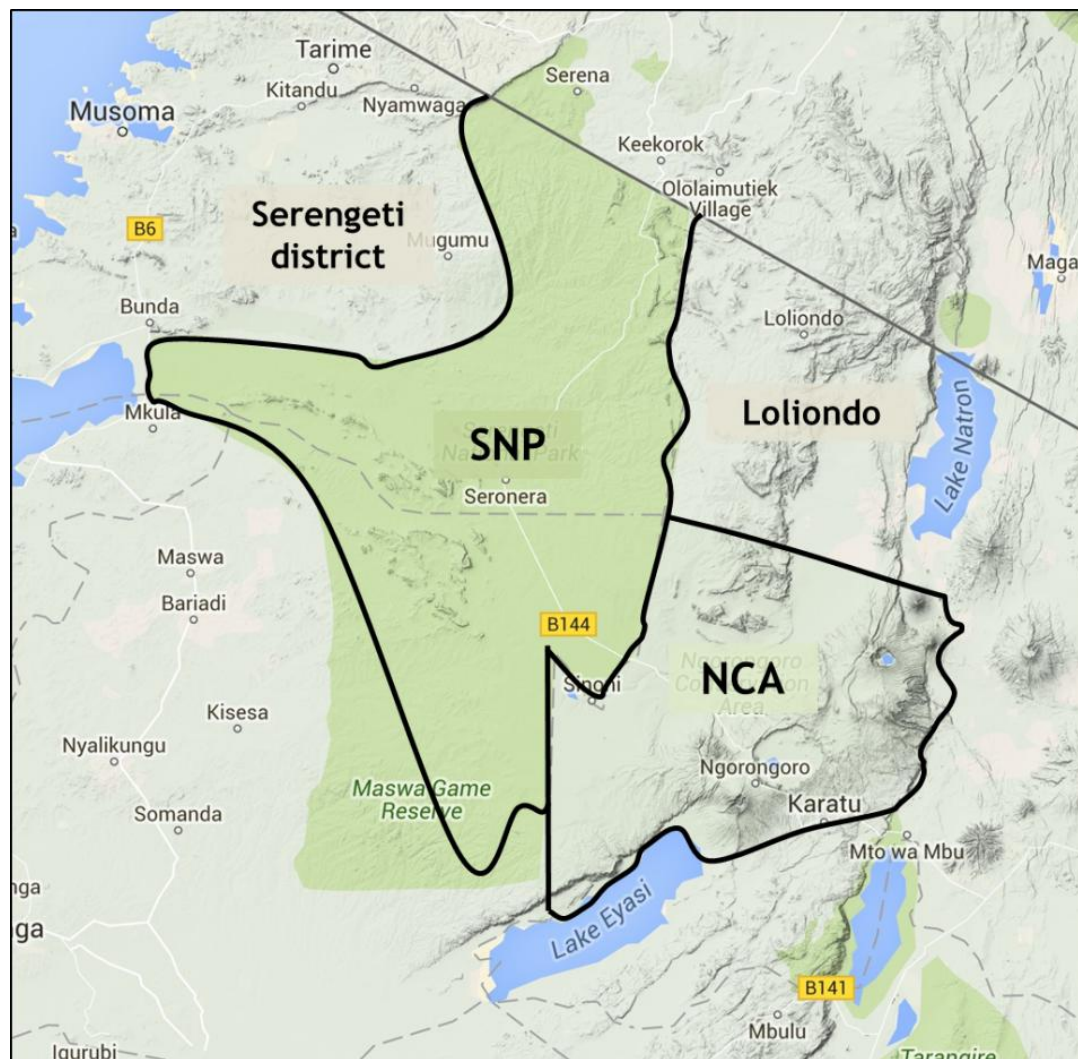


Figure 6.1 Map of Northern Tanzania demonstrating the locations of the Serengeti National Park and Ngorongoro Conservation Area. Map of North East Tanzania, thick black lines demonstrate the boundaries of the SNP and NCA (boundaries were reproduced from Somers and Hayward (2012)). Maps were produced in Google My Maps (Google, 2016).

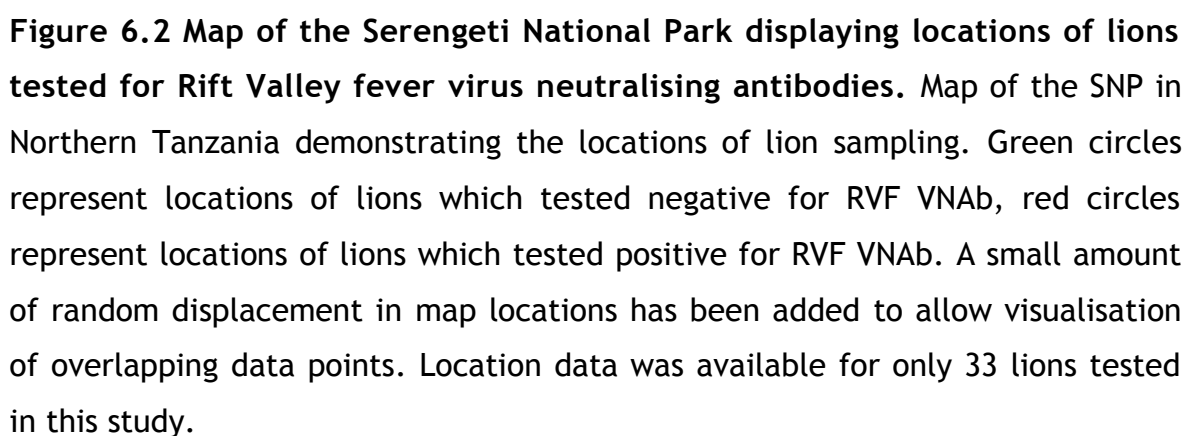
6.2 Results

6.2.1 Rift Valley fever virus neutralising antibodies in lions from the Serengeti National Park

We had access to lion samples which had been opportunistically collected as part of management or research interventions in the SNP between 2008 and 2011. Sera had been collected from lions within, or close to the border of the SNP (Figure 6.2). These sera were considered an interesting sample set to test for RVF VNAb, as a previous serological survey had demonstrated antibodies in African lions (House et al., 1996). To investigate whether these lions had been exposed to RVFV, 36 samples were tested on the RVFV-luc NT.

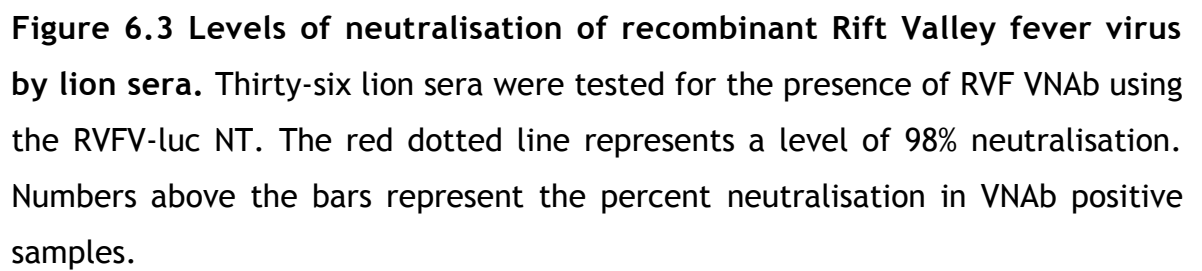
The number of samples collected per year was fairly evenly distributed, however sampling was highly skewed towards female lions (88%) (Table 6.1). Age data recorded for the lions was approximate, estimated on the basis of nose colouration (Whitman et al., 2007), and was typically recorded as adult (3+ y), young adult (18 m - 3 y) or young (<18 m). Thirty-three of the lions tested were classified as adults, two as young adults and one as young.

Twelve lion samples tested positive for RVF VNAb, with positive samples observed in all years included in the study (Figure 6.3). The overall seroprevalence in these lions was 33% (95% CI 19-50%). Eleven of the positive samples were from adult lions, and one positive result was obtained from the only 'young' lion among the sampled individuals. The sample from the young lion was collected in November 2010, 3 years following the end of the 2006/2007 RVFV outbreak, this lion would therefore have lived only through IEP. The results of our study support previous evidence of RVFV exposure in African lions and additionally provides evidence of inter-epidemic exposure and seroconversion to RVFV in lions in the Serengeti ecosystem.



Year	M	F	NK	Total
2008	-	6 [1]	-	6 (17%)
2009	-	7 [3]	-	7 (43%)
2010	4 [1]	6 [3]	-	10 (40%)
2011	-	9 [3]	1 [1]	10 (40%)
NK	-	1 [0]	2 [0]	3 (0%)
Total	4 (25%)	29 (34%)	3 (33%)	36 (33%)

Table 6.1 Sample distribution of lion samples tested for Rift Valley fever virus neutralising antibodies. Lion samples from the Serengeti National Park in Tanzania, collected between 2008 and 2011, were tested for VNAb using the RVFV-luc NT. Numbers in square brackets represent the number of lions testing positive for RVF VNAb. Numbers in brackets represent the percentage of animals testing positive for VNAb. NK = not known.



6.2.2 Rift Valley fever virus neutralising antibodies in African buffalo and gazelle in the Serengeti National Park and Ngorongoro Conservation Area

In this study 173 serum samples from African buffalo (n=99) and gazelle (n=74) from within the SNP or from NCA were tested for RVF VNAb using the RVFV-luc NT. Samples had been collected during wildlife disease surveillance, research and conservation management and were available from an archived serum bank. This study included 34 samples (21 African buffalo and 13 gazelle) that had previously been tested during validation of the RVFV-luc NT in Chapter 5.

Gazelle samples comprised samples from both Thomson's (n=63) and Grant's gazelle (n=11) collected between 1993 and 2012. Four of the 74 samples tested positive for RVF VNAb, providing an overall seroprevalence in gazelle of 5% (95% CI 2-13%). All seropositive samples derived from animals sampled in the NCA in 2004 and included both Thomson's and Grant's gazelles (Table 6.2), however no samples were available for testing from gazelles in the SNP at this time point. Overall, seroprevalence was significantly higher in gazelles in the NCA, 13% (95% CI 4-29%) compared to those in the SNP 0% (95% CI 0-10%) ($\chi^2=4.8$, n=68, df=1, p=0.029). No age data were available for the seropositive Thomson's gazelle, however of the seropositive Grant's gazelles, two were classified as young and the remaining animal was classified as a sub-adult in 2004, hence all three animals would have lived only through IEP.

	Ecosystem						
	Serengeti		Ngorongoro		Unknown		
Year	Thomson	Grant	Thomson	Grant	Thomson	Grant	Total
1993	-	-	-	-	6 [0]	-	6 [0]
1994	3 [0]	-	-	-	-	-	3 [0]
2004	-	-	7 [1]	6 [3]	-	-	13 [4]
2006	3 [0]	-	-	-	-	-	3 [0]
2007	1 [0]	5 [0]	-	-	-	-	6 [0]
2010	2 [0]	-	-	-	-	-	2 [0]
2012	22 [0]	-	19 [0]	-	-	-	41 [0]
Total	31 (0%)	5 (0%)	26 (4%)	6 (50%)	6 (0%)	0	74 (5%)

Table 6.2 Distribution of Thomson's and Grant's gazelle samples tested for Rift Valley fever virus neutralising antibodies. Thomson's and Grant's gazelle samples from the SNP and NCA, collected between 1993 and 2012, were tested for VNAb using the RVFV-luc NT. The numbers in square brackets represent the number of gazelle testing positive for VNAb. The numbers in brackets represent the percentage of animals testing positive for RVF VNAb.

Ninety-nine African buffalo samples, collected between 2007 and 2012, from animals in the SNP (n=49) and NCA (n=50) were tested for RVF VNAb. Thirty-two of the 99 samples were positive for VNAb, providing an overall seroprevalence of 32% (95% CI 23-43%) (Table 6.3). The seroprevalence in African buffalo from the NCA was significantly greater (46%, 95% CI 32-61%) than for buffalo from the SNP (18%, 95% CI 9-32%), ($X^2=8.6$, n=99, df=1, p=0.029).

Age data, determined from tooth eruption and horn growth and size (Sinclair, 1977) were available for 74% (n=73) of buffalo tested in this study. Using this data we calculated the RVFV age seroprevalence in these buffalo. Due to small sample sizes in each age class, data had to be combined for analysis (Table 6.4).

Seropositivity was significantly associated with age ($X^2=11.9$, $n=73$, $df=5$, $p=0.036$), with seroprevalence generally increasing with increasing age. The seroprevalence of animals which had lived through the 2006/2007 outbreak was 39% (95% CI 25-54%) and for those born after the outbreak, seroprevalence was 8% (95% CI 1-27%). Samples from two young buffaloes (3 and 4 years old) collected in 2012 were seropositive, providing evidence of infection during the IEP. As buffalo in this study were wild, age data is only an approximation, however the accuracy of tooth eruption in young buffalo (<5 years of age) is reported to be ± 0.5 years (Grimsdell, 1973) and therefore we are confident that VNAb in these two African buffalo demonstrates evidence of inter-epidemic transmission of RVFV.

Year	Ecosystem		Total
	Serengeti	Ngorongoro	
2007	13 [6]	-	13 (46%)
2010	8 [0]	-	8 (0%)
2011	15 [3]	20 [13]	16 (46%)
2012	13 [0]	30 [10]	43 (23%)
Total	49 (18%)	50 (46%)	99 (32%)

Table 6.3 Distribution of African buffalo samples tested for Rift Valley fever virus neutralising antibodies. African buffalo samples from the SNP and NCA, collected between 2007 and 2012, were tested for VNAb using the RVFV-luc NT. The numbers in square brackets represent the number of animals testing positive for VNAb, the numbers in brackets represent the percentage of animals testing positive for RVF VNAb.

Age	Number positive/tested	Seroprevalence
<3 y	0/8	0%
3-4 y	4/22	18%
5-6 y	3/14	21%
7-8 y	1/4	25%
9-10 y	6/10	60%
>10 y	7/15	47%
Total	21/73	29%

Table 6.4 Age seroprevalence of Rift Valley fever virus in African buffalo.
 Seroprevalence of Rift Valley fever virus in African buffalo when classified by age (n=73).

To compare the serology results obtained using the RVFV-luc NT, the buffalo samples tested in this study were also tested by RVFV recN indirect IgG ELISA (Paweska et al., 2007). Ninety-eight of the African buffalo samples tested by the RVFV-luc NT were also tested by ELISA. One sample was not tested due to an inadequate volume of serum available. The results of the RVFV-luc NT and the ELISA were consistent for the majority of samples (85%) (Table 6.5). Cohen's kappa was employed which reported a good agreement between both assays, $\kappa=0.64$ (95% CI 0.48-0.81).

		RVF indirect IgG ELISA	
RVFV-luc NT		Positive	Negative
	Positive	23	8
	Negative	7	60

Table 6.5 Rift Valley fever virus serology results of African buffalo when tested by RVFV-luc NT and ELISA. Ninety-eight African buffalo samples were tested for RVFV antibodies by the RVFV-luc NT and the RVFV recN indirect IgG ELISA and categorised as positive or negative. Cut-off values for seropositivity for the RVFV-luc NT was $\geq 98\%$ and for the RVFV recN IgG indirect ELISA it was ≥ 31.0 . RVFV recN indirect IgG ELISA was kindly performed by Dr. Ayman Samman (University of Glasgow).

6.2.3 Rift Valley fever virus neutralising antibodies in domestic ruminants in Northern Tanzania

In 2009 a longitudinal study of cattle, sheep and goats from villages surrounding the SNP and NCA was performed to determine the seroprevalence of RVFV antibodies in these species. The project was intended to provide baseline seroprevalence data to inform models used to predict RVFV outbreaks based on

climate and rainfall data. A selection of samples were initially tested for RVFV antibodies using commercial ELISAs, however the authors of the study experienced poor repeatability using such assays, therefore this sample set was never fully tested. With the development of the RVFV-luc NT, we were able to test these samples for RVF VNAb.

Three sets of samples were collected at four month intervals, with initial samples (time point 1) collected between October and December 2009, the second samples (time point 2) were collected between February and March 2010 and final samples (time point 3) were collected in June and July 2010. A total of 1348 animals were recruited into the study, this cohort comprised of 697 cattle, 444 goat and 207 sheep (Table 6.6). Livestock sampling was conducted in two areas; the Serengeti district to the west of the SNP, and Loliondo district to the East of the SNP and to the north of the NCA (Figure 6.4). Livestock samples were collected from eight villages in each area with three herds being sampled in each village. The majority of herds were of mixed species although a handful of these herds were comprised solely of cattle or goats. A total of 646 and 702 animals were recruited from the Serengeti and Loliondo districts respectively.

Due to time constraints we were unable to test samples from all three time points, therefore we chose to test samples from time points 1 and 3 only, allowing for the most time to have passed between sampling.

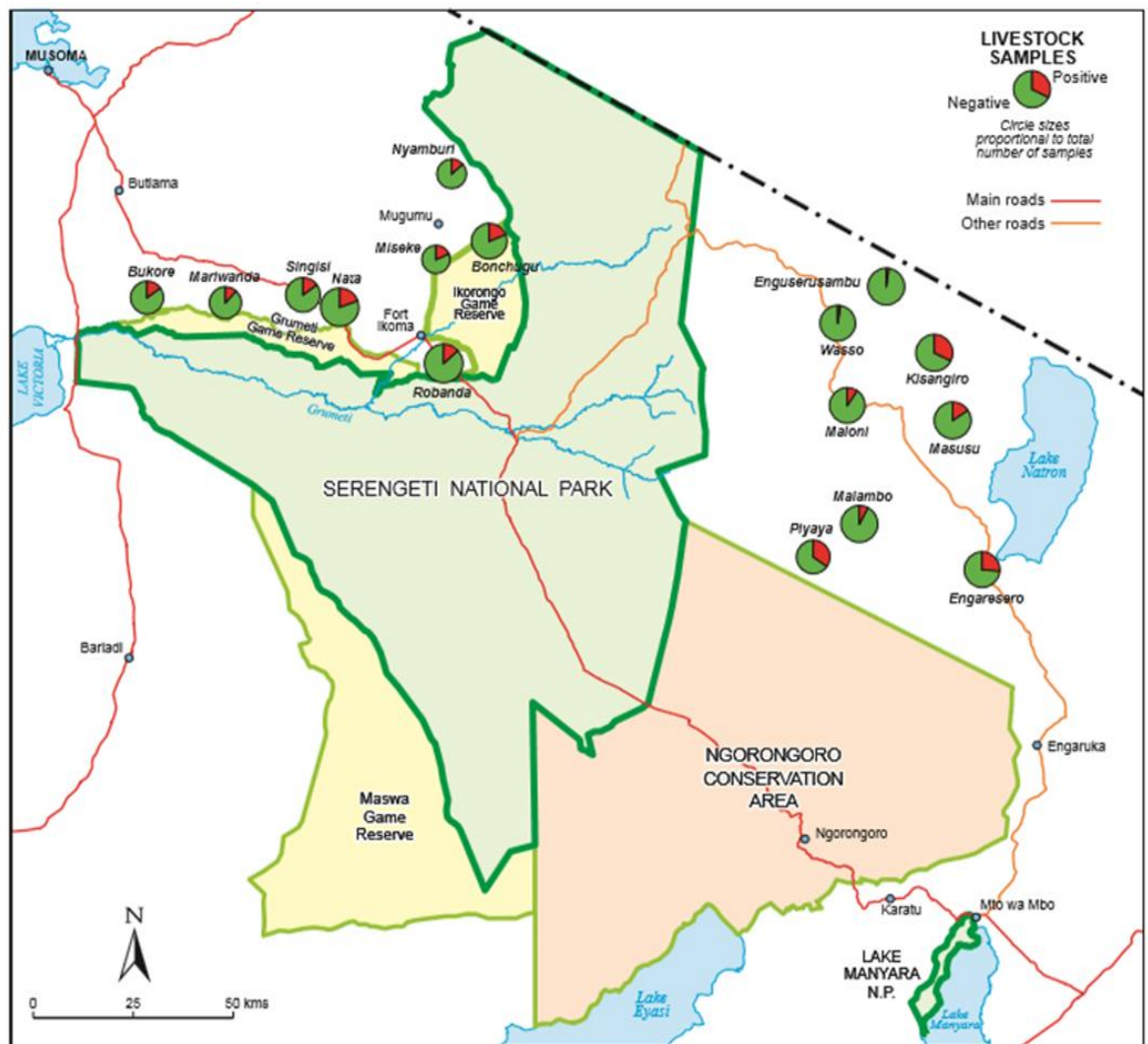


Figure 6.4 Map of villages in the Serengeti and Loliondo districts where livestock were recruited for a Rift Valley fever virus serosurveillance study. Map of the areas surrounding the SNP and NCA, demonstrating locations of villages included in the study. Pie charts represent the proportions of animals testing RVF VNAb positive (red) and negative (green) in each village at tp 1. Size of the circles is proportional to the number of samples tested in each village.

District	Village	Species	Number of animals	Total animals per village
Serengeti	Bonchugu	Cattle	57	87
		Sheep	10	
		Goat	20	
	Bukore	Cattle	35	83
		Sheep	21	
		Goat	27	
	Mariwanda	Cattle	54	76
		Sheep	0	
		Goat	22	
Serengeti	Miseke	Cattle	36	56
		Sheep	0	
		Goat	20	
	Nata	Cattle	67	101
		Sheep	4	
		Goat	30	
	Nyamburi	Cattle	41	65
		Sheep	6	
		Goat	18	
Loliondo	Robanda	Cattle	47	98
		Sheep	20	
		Goat	31	
	Singisi	Cattle	29	80
		Sheep	19	
		Goat	32	
	Engaresero	Cattle	44	90
		Sheep	15	
		Goat	31	
Loliondo	Enguserusambu	Cattle	45	90
		Sheep	19	
		Goat	26	
	Kisangiro	Cattle	43	90
		Sheep	9	
		Goat	38	
	Maaloni	Cattle	48	93
		Sheep	17	
		Goat	28	
Loliondo	Malambo	Cattle	38	90
		Sheep	20	
		Goat	32	
	Masusu	Cattle	45	90
		Sheep	15	
		Goat	30	
	Piyaya	Cattle	29	75
		Sheep		
		Goat		

	Sheep	15	
	Goat	31	
	Cattle	39	
Wasso	Sheep	17	84
	Goat	28	
Total			1348

Table 6.6 Livestock enrolled in the Rift Valley fever virus serosurveillance study. Cattle, sheep and goats from 16 villages from either the Serengeti (n=8) or Loliondo districts (n=8) of Tanzania were enrolled in a RVFV serosurveillance study in 2009.

Samples collected at the first time point (n=1302) were tested for VNAb using the RVFV-luc NT. A total of 207 animals demonstrated VNAb, providing an overall seroprevalence of 16% (95% CI 13-19%) (Table 6.7). Thirty-seven of the 44 study herds (84%) included at least one animal with RVF VNAb, three of the seronegative herds were from villages within the Serengeti district and 4 were from villages in Loliondo district. Individual village seroprevalence for villages in the Serengeti area ranged from 12% to 20% and for villages in Loliondo this ranged from 2% to 35%. Villages Enguserusambu and Wasso, which are close to the Kenyan border, had the lowest seroprevalence with only 2 seropositive animals in each village (Figure 6.4).

There was no significant difference in seroprevalence between species, with 16% of cattle (95% CI 13-19%), 14% of sheep (95% CI 9-19%) and 18% of goats (95% CI 14-22%) seropositive ($\chi^2=1.4$, n=1302, df=2, p=0.49). Cattle and goats had the highest seroprevalence in villages in the Serengeti region whereas sheep and goats had the highest seroprevalence in Loliondo (Table 6.7). However this may be influenced by the age distribution of livestock in each area, as there were a larger proportion of older cattle in villages from the Serengeti region than in Loliondo and higher numbers of sheep and goats aged over 5 years in Loliondo than in the Serengeti villages (Table 6.7).

The 2006/2007 RVFV outbreak in Tanzania occurred over two years prior to commencement of this study, therefore livestock in this study classified as less

than one year of age would have lived through IEP only. Livestock of one year or younger comprised 25% of the total number of seropositive animals in the study, strongly suggesting RVFV is present during IEP in both the Serengeti and Loliondo regions. The seroprevalence of animals that had lived through the 2006/2007 outbreak (n=600) was 17% (95% CI 14-20%) and for those born after the end of the outbreak (n=700) the seroprevalence was 15% (95% CI 13-18%).

District	Species	Age					Total
		<6 months	6-12 months	1-5 years	>5 years	Unknown	
Serengeti	Cattle	34 (12%)	56 (7%)	152 (19%)	110 (22%)	1 (0%)	353 (17%)
	Sheep	3 (0%)	29 (10%)	43 (7%)	2 (0%)	0 (0%)	77 (8%)
	Goat	35 (11%)	57 (18%)	80 (20%)	11 (9%)	1 (0%)	184 (17%)
	Total	72 (11%)	142 (12%)	275 (17%)	123 (20%)	2 (0%)	614 (16%)
Loliondo	Cattle	5 (20%)	94 (11%)	157 (15%)	68 (15%)	0	324 (14%)
	Sheep	3 (0%)	24 (17%)	77 (18%)	21 (19%)	0	125 (18%)
	Goat	1 (0%)	45 (24%)	145 (16%)	48 (21%)	0	239 (18%)
	Total	9 (11%)	163 (15%)	379 (16%)	137 (18%)	0	688 (16%)

Table 6.7 Prevalence of Rift Valley fever virus neutralising antibodies in livestock in Tanzania. Age, species and location data of 1302 livestock samples tested for RVF VNAb, collected in 2009 from either the Serengeti or Loliondo districts of Tanzania.

A generalised mixed-effects model was used to investigate whether species, age and sex were associated with seropositivity to RVFV. Seropositivity at time point one was used as the response variable and was assessed as a binary value. Species, sex and age were considered as fixed effect explanatory variables. Individuals sampled from the same location did not represent statistically independent data points, therefore to reflect this, the district, village and herd of sampled individuals were accounted for using a nested random effect. Models including interactions between fixed effects did not converge and therefore could not be properly assessed. All animals with complete information for all variables were included in the model (n=1300).

Using a backward elimination procedure, nested models were compared using likelihood ratio tests to determine which of the fixed effects should be included in the final model (Table 6.8). Model selection indicated that seropositivity to RVFV was significantly lower in males compared to females ($p=0.023$). 13% (95% CI 9-16%) of male animals demonstrated VNAb compared to 17% (95% CI 15-20%) of females. Previous studies of RVFV seroprevalence in domestic livestock have also demonstrated higher seroprevalence rates in females than males (Sumaye et al., 2013, Ringot et al., 2004).

Individual generalised linear models were used to further elucidate the relationship between seropositivity and sampling location. No difference in seropositivity rates were observed between the Serengeti and Loliondo districts ($p=0.96$), however at the village and herd level there were highly significant associations between seropositive animals and the village ($p<10^{-10}$) or herd ($p<10^{-12}$) they were sampled from.

Model	AIC	Log Likelihood	Degrees of freedom	p-value
<i>D/V/H + <u>S</u> + Sx + A</i>	1104.0	-544.0	8	0.773
<i>D/V/H + Sx + <u>A</u></i>	1100.5	-544.2	6	0.139
<i>D/V/H + <u>Sx</u></i>	1100.7	-545.3	5	0.023
<i>D/V/H</i>	1103.8	-547.9	4	1.08 x10 ⁻⁹
Intercept model	1142.5	-570.2	1	-

Table 6.8 Values associated with model selection used to derive the model of seropositivity to Rift Valley fever virus. Model formulae demonstrate variables included in the model (D=district, V=village, H=herd, S=species, Sx=sex, and A=age). Italics are used to indicate random effects while other terms were fixed effects, forward slashes indicate a nested model structure. For each model, AIC, log likelihood and degrees of freedom used in the model are shown. p-values are shown for the emboldened and underlined term in each model. These were derived from likelihood ratio tests between the model shown inline and the nested model below.

At time point 3 a total of 773 samples were collected from animals originally enrolled in the study, the reasons given by farmers for the remaining animals being unavailable for sampling included the animal dying, being moved away, slaughtered, returned to its owner or being sold. A total of 617 of the 773 samples were available for RVF VNAb testing in our study, representing 12 of the original 16 villages included in the study. A small number of samples (n=34) collected at time point 3 were from animals which were not tested for RVF VNAb at time point 1 (Table 6.9).

		Tested at time point 3 (June-July 2010)		Total
		Yes	No	
Tested at time point 1 (Oct-Dec 2009)	Yes	583	719	1302
	No	34	0	34
Total		617	719	1336

Table 6.9 Samples tested for Rift Valley fever virus neutralising antibodies at time points one and three. The number of samples tested from each time point in this study. A total of 1919 samples, from 1336 animals, were tested in this study. These numbers represent only samples used in this study and do not represent the number of samples collected in the original study.

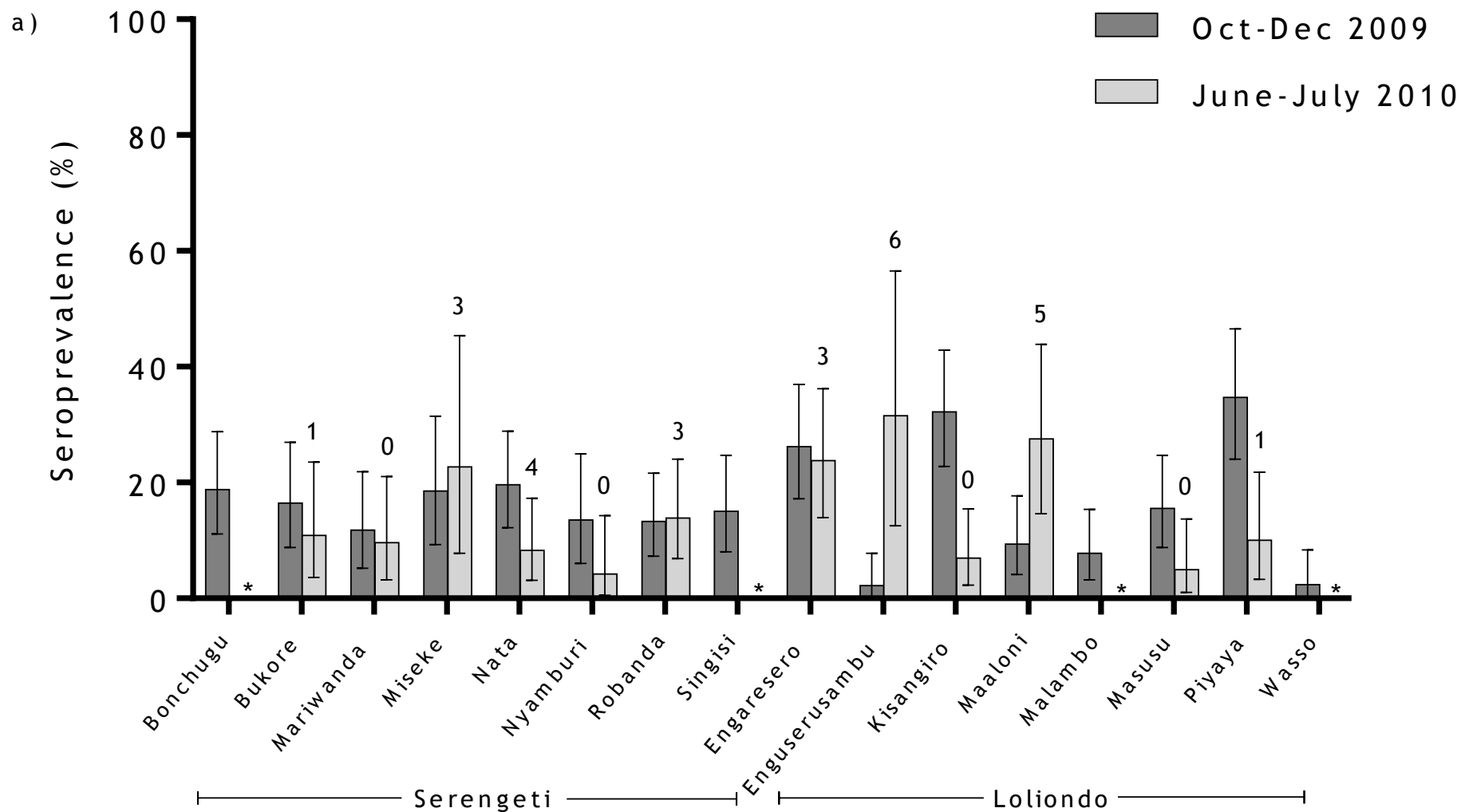
At time point 3 the seroprevalence was 11% (95% CI 7-15%) in the Serengeti district and 15% (95% CI 11-19%) in Loliondo district. In the 6-8 months since initial testing, 26/457 individuals (6%, 95% CI 4-8%) showed evidence of seroconversion to RVFV. Animals that seroconverted (cattle n=14, goat n=7, sheep n=5) during the study period came from 8 of the 12 villages tested at time point 3. The individual village level seroprevalence for both time points is presented in Figure 6.5, and there are contrasting seroprevalence rates of RVF VNAb in some villages at the first and final time points.

We further examined these longitudinal data to investigate the RVFV antibody status of individual animals at both time points (Table 6.10a). Of the 583 animals tested at both time points, the majority of animals that tested negative at time point 1 were also negative at time point 3. Of the 126 animals which

were seropositive at time point 1 only 39% remained VNAb positive at time point 3. Animals that were VNAb positive at time point 1 and then became seronegative were termed 'seroreverters'. We compared the characteristics of the animals depending on their RVF VNAb status throughout the study, analysing age, species, sex and whether the samples came from the Serengeti and Loliondo districts (Table 6.11).

To statistically investigate predictors of seroconversion and seroreversion, generalised mixed-effects models were utilised. A model was used to determine whether species, sex, or age could be used as predictors of seroconversion or seroreversion during the study period. Seroconversion and seroreversion were considered binary response variables that were modelled with a binomial error structure. As in the seropositivity model (Table 6.8), sampling location was accounted for using a nested random effect. Neither species, sex nor age was found to correlate significantly with seroconversion or seroreversion. Separate generalised linear models were used to further elucidate the relationship between antibody status and sampling location. In both seroconversion and seroreversion models, village ($p < 0.0001$) and herd ($p < 0.005$) were statistically associated with serostatus, however district was not.

The ROC analysis in Chapter 5 suggested a cut-off value of 94% for the RVFV-luc NT, however we opted for a more stringent cut-off of $\geq 98\%$ to ensure optimum specificity of the assay, perhaps at the expense of sensitivity (sensitivity=94% and specificity=100%). To investigate whether the same serological patterns were observed using the lower cut-off of $\geq 94\%$, we compared the RVF VNAb status of samples tested at both time points using both test cut-offs (Table 6.10), and similar levels of seroreversion were demonstrated.



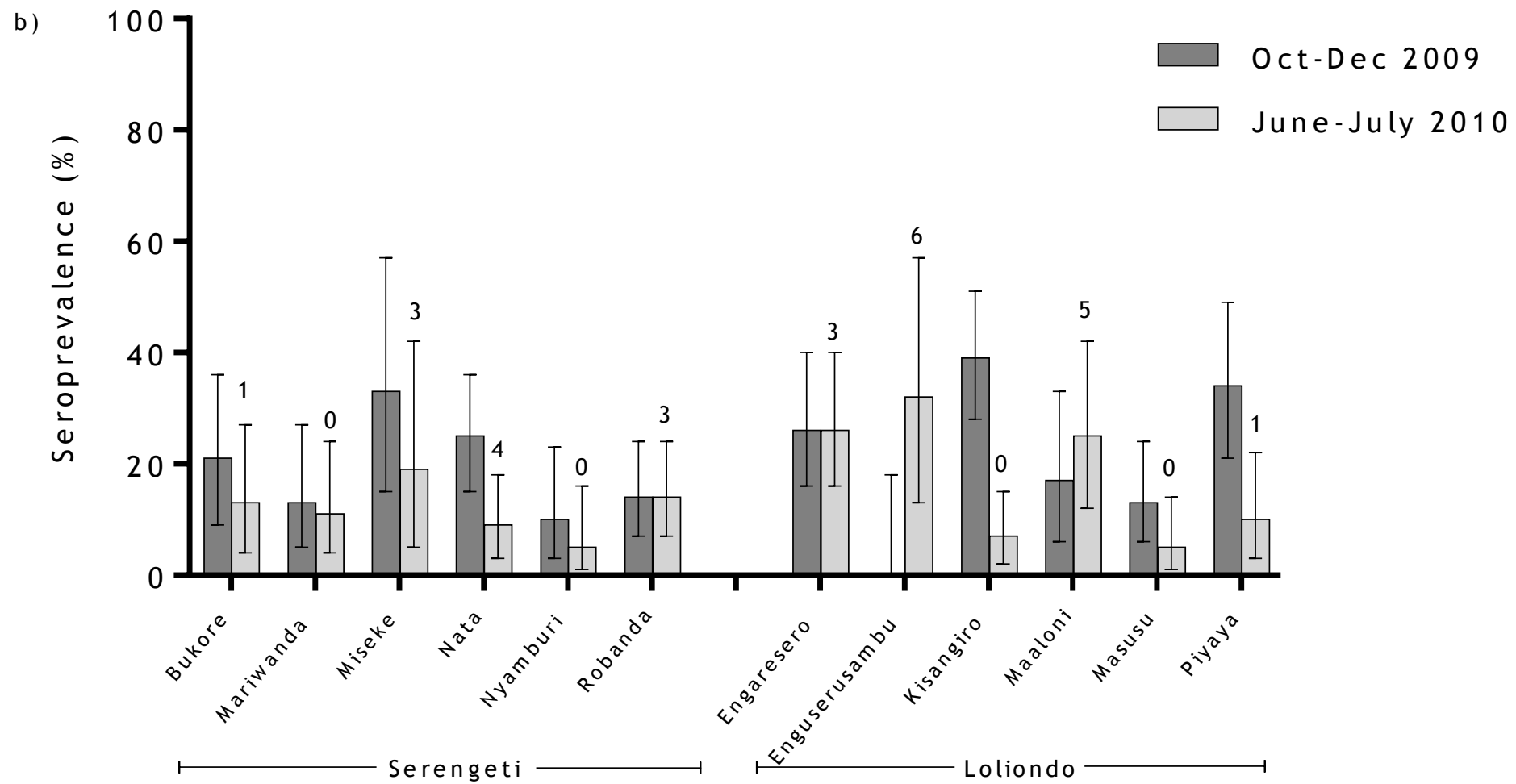


Figure 6.5 Village level seroprevalence of Rift Valley fever virus neutralising antibodies at time point one and time point 3. Livestock from villages in the Serengeti and Loliondo districts were tested for RVF VNAb and seroprevalence rates were calculated for time point 1 (Oct-Dec 2009) (n=1302) and time point 3 (June-July 2010) (n=617). *represents villages that were not tested at time point 3. The numbers above June-July 2010 seroprevalence bars indicate the number of animals in that village which seroconverted to RVFV between time points one and three. Error bars represent 95% confidence intervals. a) Includes all animals tested at each time point (n=1919) b) includes only animals tested at both time points (n=583).

a)		≥98% cut-off		b)		≥94% cut-off	
		Time point 3				Time point 3	
		NEG	POS			NEG	POS
Time point 1	NEG	431 (94%)	26 (6%)	Time point 1	NEG	335 (85%)	57(15%)
	POS	77 (61%)	49 (39%)		POS	104 (54%)	87 (46%)

Table 6.10 Comparison of Rift Valley fever virus antibody status of livestock tested at both time points using two assay cut-off values. Longitudinal data (n=583) was analysed using two cut-off values for the RVFV-luc NT test a) $\geq 98\%$ and b) $\geq 94\%$.

		Sero-reverters	Consistent seropositives	Consistent seronegatives	Sero-converters
Age	<1 y	16%	6%	16%	15%
	1-5 y	61%	65%	73%	81%
	5+y	23%	29%	11%	4%
Species	Cattle	57%	51%	61%	54%
	Sheep	16%	9%	29%	19%
	Goat	27%	40%	10%	27%
Sex	Female	79%	86%	71%	77%
	Male	21%	14%	29%	23%
District	Serengeti	40%	43%	48%	42%
	Loliondo	60%	57%	52%	58%

Table 6.11 Characteristics of animals classified by Rift Valley fever antibody status at time point 3. Animals which tested RVF VNAb positive at time point 1 and became RVF VNAb negative at time point 3 were termed ‘seroreverters’ (n=77), those which remained VNAb positive at time point 3 were termed ‘consistent seropositives’ (n=49). Animals which seroconverted between the two time points were termed ‘seroconverters’ (n=26) and those which remained seronegative throughout the study were termed ‘consistent seronegatives’ (n=431). The age, sex, species and district of animals in both groups were compared.

6.3 Discussion

Rift Valley fever virus has devastating effects on the economy, human health and animal health and production during outbreaks. Active surveillance is required during IEP to improve predictions, and the early detection, of RVFV outbreaks. Currently the majority of RVFV surveillance in Tanzania is passive, relying on communities who have little contact with veterinarians reporting potential RVFV cases. Additionally, the symptoms of RVFV are similar to other diseases such as peste des petits ruminants virus and brucellosis, and outwith RVFV epidemics the virus is not always considered by farmers as a cause of disease (Wensman et al., 2015). Robust serological assays are required to allow for active serosurveillance. The RVFV neutralisation test developed in Chapter 5 allows for high-throughput detection of RVF VNAb from sera, of any species, with results available in less than 8 hours.

These studies clearly demonstrate the value of applying the RVFV-luc NT to the investigation of the multi-host epidemiology of RVFV in a complex, natural ecosystem. Although inter-epidemic circulation of RVFV has been documented previously in Southern Tanzania (Sumaye et al., 2013, Sumaye et al., 2015) and elsewhere in East Africa (Evans et al., 2008), these studies add to our understanding of spatial and temporal patterns of RVFV circulation. The key findings of these epidemiological studies include 1) evidence of infection in a wide range of mammalian host species, including several wildlife (lions, buffalo and gazelles) and livestock species (cattle, sheep and goats); 2) robust support for the existence of inter-epidemic circulation of RVFV; 3) evidence of high rates of seroreversion in livestock, suggesting a rapid decline in VNAb titres in villages with no or low levels of recent infection.

In this study we demonstrated a high RVFV seroprevalence in lions, a species that has been scarcely mentioned in the RVFV literature. The only published account of RVF VNAb in lions documented seropositivity in 56% (9/16) of lions from Ngorongoro and 10% (3/31) of lions from the Kruger National Park, South Africa (House et al., 1996). In their study, samples from lions, African wild dogs and cheetahs were tested for RVF VNAb with only lions demonstrating seropositivity. Additionally, a large unpublished study on RVFV serosurveillance in lions from the SNP, performed by Prof. Craig Packer (University of Minnesota),

also documented RVF VNAb in lions. In his study 240 lion serum samples, collected between 1984 and 1999, were tested for RVF VNAb using the VNT. Samples collected between 1984 and 1997 all had titres of ≤ 40 , whereas 40% of lions sampled in 1998 and 1999 were seropositive (Packer, 2016, unpublished data), possibly suggesting infection in lions after the RVFV outbreak in Tanzania in 1997/1998. Clinical disease has not been reported in lions, either due to RVFV causing only subclinical infections or as it is not considered a cause of disease in this species. There have been many reports of lions being infected with pathogens not primarily associated with wild felids, such as canine distemper virus, rabies and bovine tuberculosis (Roelke-Parker et al., 1996, Berentsen et al., 2013, Renwick et al., 2007), hence RVFV infection in lions would not come as a complete surprise. Lions may become infected with RVFV either directly via the bite of an infected mosquito, or by ingestion of infected prey, such as gazelle and buffalo; species which demonstrated evidence of infection in our study.

Our finding of seropositivity in buffalo is consistent with previous studies demonstrating high prevalence of antibodies to RVFV in African buffalo from Kenya (Evans et al., 2008), South Africa (LaBeaud et al., 2011, Beechler et al., 2015), Botswana (Jori et al., 2015) and Tanzania (Sindato et al., 2013). Reports of antibodies to RVFV in gazelle have also been previously reported (Britch et al., 2013, Evans et al., 2008), however this study is, to our knowledge, the first to report seropositivity in Grant's gazelle. With 75% of seropositive individuals this species, further investigation into the role of Grant's gazelle in the epidemiology of RVFV is clearly warranted. Clinical symptoms of RVFV infection have been observed in both buffalo and gazelle (Beechler et al., 2015, ProMED-mail, 2013) and additional research is required to further elucidate the role of these wildlife species in RVFV maintenance.

In this study we detected RVF VNAb in 16% of domestic livestock tested. Seroprevalence studies from other regions of Tanzania have demonstrated RVFV seroprevalence of 40% in livestock during outbreaks (Chengula et al., 2014) and 8-11% seropositivity outwith outbreaks (Wensman et al., 2015, Sumaye et al., 2013). Additionally, RVFV antibodies have been demonstrated in livestock from regions of the country that have never experienced RVFV outbreaks (Kifaro et al., 2014).

In both gazelle and buffalo the number of RVF VNAb positive animals was higher in wild ruminants from NCA compared to those from the SNP. The NCA and Loliondo district are considered high risk areas for RVFV, with outbreaks recorded historically in this area during many of the previous epidemics (Sindato et al., 2014). However the precise risk factors are still uncertain and it is unknown whether the same risk factors that predispose to an epidemic during periods of high rainfall are the same as those that predispose to infection during IEP. An interesting observation from this study was that, unlike wild ruminants, the seroprevalence of livestock was similar in both the Serengeti and Loliondo districts and further analysis will be required to identify risk factors in the different regions.

Numerous gaps still remain in understanding the epidemiology of RVFV during IEP. The leading hypothesis suggests RVFV does not circulate between outbreaks, but is instead maintained in mosquito eggs. However, there is now a growing body of evidence suggesting RVFV is indeed circulating and being maintained in mammalian hosts between outbreaks. In this Chapter we observe VNAb in animals, of all species tested, which have lived only through IEP. The longitudinal data also reveals geographically widespread infection during IEP which has seldom been documented in previous serosurveys as these have tended to concentrate on single areas. A limitation of the studies presented in this Chapter, and the use of VNTs in general, is that we cannot comment on the recency of infection, which can only be inferred by use of IgM ELISA, or directly via virus detection. However, as we have accurate age data for the majority of animals in our studies, we can be confident that the data presented here represents evidence of inter-epidemic transmission of RVFV in Northern Tanzania.

Previous studies have also reported RVFV antibodies in animals which have lived only through IEP, these include both livestock (Sumaye et al., 2013, Lichoti et al., 2014, Rostal et al., 2010, Wensman et al., 2015) and African buffalo (Beechler et al., 2015), and further studies have observed the same in humans (LaBeaud et al., 2008, Sumaye et al., 2015, Gray et al., 2015). The results presented in this Chapter provide strong evidence for widespread, low-level circulation of RVFV in a multi-host ecosystem during IEP.

In the longitudinal study of livestock we observed a high proportion of RVF VNAb positive animals whom were antibody negative when re-tested 6-8 months later. These results were unexpected, as in the literature many describe RVF VNAb as being lifelong (Lwande et al., 2015, Jori et al., 2015, Pepin et al., 2010, Paweska et al., 2005). However, Niklasson et al. (1984) demonstrated a peak in RVF VNAb around 6 weeks post infection which dropped significantly to around a titre of 1:40 at 26 weeks post infection, furthermore very little data is available on the duration of RVF VNAb in natural infection. It is unknown whether animals that were previously VNAb positive, but now have undetectable antibody titres would still be provided protection from infection.

A small proportion of animals that were less than one year of age at time point 1 may have demonstrated seroreversion during the study period due to the loss of maternal RVFV antibodies, as maternal antibodies wane at around 7 months of age (Sumaye et al., 2013). However, this would only explain a very small proportion of seroreversions observed in our study. We also considered that the stringent cut-off of $\geq 98\%$ on the RVFV-luc NT could have artificially elevated the number of seroreverters observed in this study, due to lowering the assay sensitivity. However, we reanalysed the results of all paired samples tested using the lower threshold of 94% and observed similar serological patterns (Table 6.10).

We hypothesise that the high proportion of animals demonstrating seroreversion may be due to waning of antibody after natural infection. As the date of exposure in these animals is unknown however such a hypothesis is difficult to evaluate. A high proportion of seroreverters were observed in villages in which no seroconversions were detected during the study period. Additionally, statistical models revealed no correlation between species, sex or age in relation to seroreversion, however significant correlations between village and herd were demonstrated. These results may suggest maintaining detectable antibody levels may rely on constant priming of the immune system with repeated viral exposures.

Fafetine et al. (2013) performed longitudinal studies in sheep and goats in Mozambique and demonstrated 2/9 animals which were RVFV IgG positive at the beginning of the study were seronegative after 3-4 months. A longitudinal study

of African buffalo in South Africa also documented seroreversion in five buffalo over a six year study period (LaBeaud et al., 2011). In both of these studies the numbers of animals investigated was small. Our longitudinal study provides preliminary evidence of antibody waning in a cohort of over 500 naturally infected animals, which is the most comprehensive study to date. Further research is required to investigate the rate at which antibodies to RVFV wane over time. Analysing the middle time point (time point 2) from the original study, in addition to quantifying antibody titres would be essential to provide further evidence on the apparent waning of antibody observed in these animals, and this should be the initial experiment performed in any further research on this cohort. It is likely that herd immunity would significantly impact on the likelihood of an outbreak occurring and we may find that part of the complex ecology that allows explosive outbreaks to occur, includes the depletion of RVFV herd immunity.

Many of the seroprevalence studies in the literature utilise commercially available ELISAs to detect antibodies to RVFV, such ELISAs have been shown to cross-react with other phleboviruses and have a high background which may cause false positive results. Owing to such high rates of false positives, some studies have used ELISAs to screen large numbers of animals for RVFV IgG and then confirm these using the VNT. Although ring trials have reported greater than 90% agreement between ELISAs and VNT, it appears to be much lower under field conditions, with correlations of 52% being reported in humans (Gray et al., 2015), 61% in buffalo (Evans et al., 2008) and 24-55% in domestic livestock (Kariithi et al., 2010, Boussini et al., 2014). In this study we tested African buffalo sera on both the RVFV-luc NT and a commercial indirect IgG ELISA and demonstrated a good correlation between the assays, however 15% of the results were discordant. Four of the 15 discordant samples had previously been tested by RVFV IgM capture ELISA, on IgM testing two samples were in agreement with results obtained from the RVFV-luc NT, one was in agreement with the RVFV IgG ELISA, and one was equivocal so could not be compared.

Such results demonstrate the variability in RVFV serology data reported depending on the test used, further supporting the essential requirement for robust and repeatable serological assays for surveillance. This further reinforces the use of the more stringent cut-off of $\geq 98\%$ in the RVFV-luc NT, to retain the

highest levels of specificity. It should however be noted that antibodies detected in the RVFV-luc NT are antibodies capable of neutralising virus, these VNAb are produced predominantly against epitopes on the viral glycoproteins, in contrast to antibodies detected in the commercial ELISAs which are raised to the viral nucleoprotein. Although natural exposure to RVFV should elicit antibodies to both viral glycoproteins and the nucleoprotein, the antibody titre and duration of these antibodies may vary.

Although we reported a sensitivity and specificity of 94% and 100% respectively for the RVFV-luc NT in Chapter 5, this data was based on a small validation panel (n=49) and the results of these serosurveys should be interpreted with this in mind. The test ideally requires validation using a larger collection of sera previously tested using the VNT, and further validation of the RVFV-luc NT is currently ongoing. Additionally, it would be preferable to screen sera on the RVFV-luc NT using a single serum dilution of 1:100 (as performed in these studies) and then further titrate out sera which test positive, to provide a quantitative VNAb titre. Given more time we would have applied this protocol to all positive samples in these serosurveys, to provide further confidence in the results presented herein.

The results of these sero-epidemiological studies establish the need for continued surveillance of RVFV in both domestic and wildlife species. Understanding the dynamics of RVFV, including IEP infection patterns, is critically important to identify potential approaches to disease control strategies in Tanzania. Integrating RVFV serology into already established disease monitoring programmes in and around the SNP would greatly improve the understanding of the disease dynamics in such a complex ecosystem. Based on the results presented herein, which support epidemiological data obtained using classical antibody detection methods, the RVFV-luc NT appears to be a reliable high-throughput alternative to the VNT.

Chapter 7. Discussion

7.1 Alternative neutralisation tests

Understanding viral disease ecology, especially for viruses in which multiple host species exist, relies heavily on serology. In this thesis I developed two assays, the rabies virus pseudotype neutralisation test (pNT) and the Rift Valley fever virus luciferase neutralisation test (RVFV-luc NT), for the detection of virus neutralising antibody (VNA_b) to these two important zoonotic viruses.

Virus neutralisation tests (VNTs) are considered the most specific assays for detecting VNA_b to rabies and Rift Valley fever virus (RVFV), with these tests being the World Organisation for Animal Health (OIE) prescribed tests for international trade. However, these VNTs use live virus to detect neutralisation, requiring them to be performed at high containment and presenting potential infection risks for laboratory staff. Both rabies and RVFV are endemic in Africa, where very few laboratories have the containment facilities necessary to perform such assays. Therefore, serosurveillance of these viruses are not readily performed in countries with the greatest need, alternatively in these countries serosurveys are performed using assays with poorer specificities.

In addition to the high containment facilities required, these assays are labour intensive, have long incubation periods, require a level of post-assay processing and are costly. In this project, I aimed to develop novel neutralisation assays which would improve on the current gold standard assays for rabies and RVFV, whilst retaining high specificities. Such improvements included negating the requirement for high containment, reducing or removing post-assay processing, decreasing the incubation time of the assay, allowing for high-throughput testing to be performed and providing economical assays for use in resource limited settings. Table 7.1 summaries the improvements achieved with the development of the rabies virus pNT and the RVFV-luc NT described in earlier chapters.

	Rabies virus		Rift Valley fever virus	
	FAVN/RFFIT	Rabies virus pNT	PRNT	RVFV-luc NT
Time to perform	24-72 h	48 h	3-7 days	8 h
Cost	High	Low	Medium	High
Post-assay processing required	Yes	No	Yes	Yes
Containment level required	BSL-3+	BSL-2	BSL-3	BSL-3 (with potential for BSL-2)
Subjectivity in interpretation	Yes	Yes	Yes	No
High-throughput	No	No (with potential for high-throughput)	No	Yes
Control for non-specificity included	No	Yes	No	No (with potential for control)

Table 7.1 Comparison of conventional virus neutralisation tests with the alternative assays developed in this study. Comparison of the main attributes of the conventional neutralisation tests used for rabies and RVFV with the rabies virus pseudotype neutralisation test (pNT) and the luciferase expressing RVFV neutralisation test (RVFV-luc NT). RFFIT=rapid fluorescent focus inhibition test, FAVN=fluorescent antibody virus neutralisation test, PRNT=plaque reduction neutralisation test, BSL=biosafety level.

The rabies virus pNT is low cost, can be performed at biosafety level (BSL) 2, requires no post-assay processing and includes a control for non-specificity. Such assays would be particularly beneficial in resource limited settings as only a fluorescence microscope is required. However, if fluorescence microscopy is unavailable the GFP reporter could easily be replaced with a *lacZ* gene, which further decreases the cost of the assay and requires no specialist equipment. An inability to identify a suspension cell line which was permissive to MLV(rabies) pseudotypes prevented analysis by flow cytometry from being further pursued. The rabies pNT is however amenable to high-throughput testing which has been successfully demonstrated for a Nipah virus pseudotype based neutralisation test (Kaku et al., 2012). Furthermore, Mather et al. (2014) have demonstrated that rabies virus pseudotypes can be lyophilised and subsequently used in a pNT, establishing the viability of a freeze-dried pseudotype-based kit to be distributed to resource limited settings.

After the success of the rabies virus pNT, similar methods were employed to produce a neutralisation test to detect RVF VNAbs, however RVFV pseudotypes were not produced to a titre that was high enough to permit their use in a pNT. The inability to produce high titre RVFV pseudotypes may be a result of the intrinsic cellular localisation of RVFV glycoproteins, which are expressed predominantly at the Golgi apparatus. The location of glycoproteins in the producer cells prior to pseudotype budding is known to affect successful incorporation of glycoproteins into pseudotype particles (Sandrin and Cosset, 2006, Whitt, 2010). In this study we did not quantitatively investigate the levels of RVFV glycoprotein expression on the cell surface, however further efforts to

improve plasma membrane expression levels should be investigated if attempts to pseudotype RVFV are pursued further. Examples of such improvements presented in the literature were discussed in Chapter 5.

As an alternative to the RVFV pNT, we developed a novel neutralisation assay utilising a recombinant luciferase expressing RVFV in place of wild type virus. The use of a single serum platform allowed for high-throughput testing to be performed using this assay. Additionally, this test required just 6 h incubation providing a considerable improvement over the plaque reduction neutralisation test (PRNT), in which up to 7 days are required to visualise viral plaques. The assay therefore provides a rapid, high-throughput alternative to the PRNT, however the use of a commercial luciferase substrate makes the assay costly in comparison, which would likely render its use prohibitive in resource limited settings. The RVFV-luc NT utilises the MP-12 strain of RVFV, which requires only BSL-2 containment in the United States (CDC, 2009), however the use of this strain still requires BSL-3 containment in the United Kingdom, restricting its use in the majority of laboratories. However, as the virus utilised in the RVFV-luc NT is a highly-attenuated strain in which the non-structural protein gene has been deleted, BSL-3 requirements for this virus may potentially be downgraded in the United Kingdom.

The data presented in the RVFV sero-epidemiological studies suggest that the RVFV-luc NT is a robust, high-throughput alternative to the PRNT. However, validation of the assay was performed on a small panel of previously tested sera. For a more accurate assessment of assay sensitivity and specificity, and to determine the most appropriate assay cut-off, a larger validation panel of sera previously tested by PRNT is required. Due to time constraints in this study it was not possible to perform a more comprehensive validation and consequently a stringent cut-off of $\geq 98\%$ was chosen as a high specificity was considered to be of most importance in our serosurveys.

To further improve the RVFV-luc NT, serum samples testing positive at a dilution of 1:100 should be further titrated to allow a quantitative titre to be obtained. In utilising such an approach the stringent cut-off could be lowered, as high sensitivity is preferable to high specificity in a screening assay, as false positives will be detected upon full serum titration. This assay holds huge potential for

large scale RVFV serosurveillance, however to ensure the most confidence in the serological data generated, further validation of the assay is necessary.

7.2 Non-specificity in neutralisation tests

The results described in this thesis are based upon testing of over 3000 sera, from a wide variety of domestic and wildlife species, for evidence of VNAb. Throughout this study we experienced serum which proved cytotoxic, demonstrated non-specific inhibition of virus infection and replication or obscured the cell monolayer. Such effects of serum have been noted in previous studies, concerning many serological tests, not only VNTs (Ananthanarayan and Paniker, 1960, Tauraso et al., 1971, Mani and Madhusudana, 2013, Bedeković et al., 2013, Shiraishi et al., 2014a).

When examining the cell monolayer visually for the presence of GFP in the rabies virus pNT, we observed examples of non-specific neutralisation and cytotoxicity, as described in Chapter 4. The integrity of the cell monolayer is critical for the interpretation of the VNT, as viruses, or pseudotypes, can only replicate/express efficiently in healthy cells. Hence, in the pNT if pseudotypes cannot transduce the cell line, and no GFP expression occurs, this could be mistakenly attributed to the presence of VNAb. To reduce the risk of such false positives occurring the cell monolayer is observed under the light microscope for evidence of cytotoxicity, however non-specific inhibitors present in serum can also inhibit pseudotype entry causing false positives even in the presence of a healthy cell monolayer.

Inclusion of the MLV(VSV) pseudotype control with each sample tested ensured that neutralisation demonstrated in the assay was due to antibody-specific neutralisation as opposed to non-specific neutralisation or inhibition. The inclusion of the MLV(VSV) control in the lyssavirus pNT was considered to be of critical importance and consequently this assay may have superior specificity than conventional VNTs. Previous studies utilising pNTs have described multiplex assays in which VNAb to one or more viruses can be detected in a single assay (Wright et al., 2010, Molesti et al., 2014). Such approaches could be used to integrate the MLV(VSV) control into a multiplex assay allowing non-specific

neutralisation to be detecting in the same well as rabies VNAb, decreasing both assay set up times and volumes of serum required. Zhou et al. (2011) have utilised a similar method to develop a dual entry pseudotype assay to detect viral entry inhibitors for SARS coronavirus (SARS-CoV) which utilises both SARS-CoV and MLV pseudotyped HIV particles. The MLV pseudotype acts as a control to detect compounds that cause non-specific inhibition.

There is currently no control for non-specific neutralisation in the RVFV-luc NT. Additionally, as the assay is analysed using a luminometer, cell monolayers are seeded in opaque, white plastic 96-well plates and therefore also cannot be examined manual for cytotoxicity. In the RVFV serosurveys presented in this chapter, serum was screened at a dilution of 1:100, hence reducing the potential contribution from any non-specific inhibitors present in sera at low dilutions. However, we cannot guarantee that a minority of these positive samples may have been the result of cytotoxicity or high levels of non-specific neutralising factors in the sera. As discussed in Chapter 5, previous studies have been described in which a metabolic viability dye is incorporated into luciferase expression assays to assess cell viability. To further improve the RVFV-luc NT, such dyes, or indeed multiplexing an unrelated virus expressing firefly luciferase for example, could be employed as indicators of non-specificity. However, vesicular stomatitis virus (VSV) may not be the ideal candidate for use as a control virus as VSV affects livestock and related vesiculoviruses have been identified in Africa (Fontenille et al., 1994, Traoré-Lamizana et al., 2001).

Multiplexing both the RVFV-luc NT and the rabies virus pNT, not only with controls for non-specificity, but also with other relevant viruses, would allow for serosurveillance of multiple viruses using one serum dilution. Such assays would be especially valuable for wildlife testing, where often only small volumes of sera are available. Examples of multiplex assays which would have been relevant in these sample sets include the multiplex analysis of VNAb to rabies and canine distemper virus in Serengeti lions and the detection of VNAb to Rift Valley fever, peste des petits ruminants and foot-and-mouth disease virus in the livestock samples from Northern Tanzania.

Virus neutralisation tests are considered the most specific assays for detecting viral induced antibodies and both assays developed in this project appear to be

as specific as their conventional counterparts. However, as demonstrated with the phylogroup one lyssaviruses even VNTs with the highest specificity, by their very nature cannot differentiate between viruses in which the glycoprotein sequences are highly similar. When interpreting VNTs, one must be aware of antigenically similar viruses circulating in the study area and additionally we must also consider the as yet uncharacterised viruses circulating globally.

7.3 Rabies virus serology

In this thesis I demonstrate rabies VNAb in lions and domestic dogs that had not previously been vaccinated against rabies virus. Rabies VNAb present due to natural infection in both animals and humans has been demonstrated in previous serological studies (Table 1.2). However, there is very little in the literature regarding the relevance of such observations, or on the clinical course of such abortive infections. Although evidence of rabies VNAb in healthy humans, domestic animals and wildlife has been demonstrated in many studies, the fate of the majority of these seropositive individuals remains unknown. In this thesis I present evidence of rabies VNAb in free-roaming dogs that were followed for 14 months and remained healthy throughout the study period.

Although the lions tested in our study were not followed over time, we are confident that none of the lions succumbed to rabies virus, as there are intense disease monitoring efforts in the Serengeti National Park (SNP), particularly in lions, many of which have been followed from birth by the Tanzania Wildlife Research Institute. Additionally, there have been no documented cases of rabies in lions in the SNP (Prof. Sarah Cleaveland, 2016, personal communication). Previous studies of other carnivore species in the SNP have also demonstrated rabies VNAb in animals in the absence of clinical disease (East et al., 2001). The route of rabies infection in lions is unknown. There have been documented cases of lions receiving bites from suspected rabid dogs, (Prof. Sarah Cleaveland, 2016, personal communication), however hunting infected prey, as suggested by Berry et al. (1993), would seem a more regular route of viral exposure. During hunting, the lions face comes into close contact with the prey's head, by means of the lion suffocating or strangling its prey, and fatal rabies infection via oral and intranasal transmissions has been demonstrated in experimental infection

(Charlton and Casey, 1979). Rabies infection via ingestion of infected material has also been documented experimentally in carnivores (Bell and Moore, 1971). However, in their study those animals which did not show signs of infection also did not demonstrate evidence of seroconversion. Delpietro et al. (1990) reported rabies induced deaths in three of 132 rodents after ingestion of rabies-infected tissues. Additionally, in their study 22 of the surviving animals demonstrated rabies VNAb in serum, strongly suggesting ingestion of infected prey could be associated with protection from lethal rabies infection.

We are confident that the rabies VNAb titres reported in previous chapters are specific, both due to the high test cut-off and the inclusion of a non-specific neutralisation control in the rabies virus pNT. We are therefore confident that the results presented herein represent evidence of natural, abortive rabies infection in both lions and free-roaming dogs. Further epidemiological evidence supports these claims, as years in which lions were found to be rabies VNAb positive correlated with years in which wildlife cases of rabies were reported in and around the SNP.

Samples in which serum dilutions of 1:40 or greater neutralise MLV(rabies) pseudotypes are considered positive for rabies VNAb in these serosurveys. This cut-off is far higher than the 1:5 dilution considered positive for rabies-specific VNAb suggested by the Advisory Committee on Immunisation Practices (ACIP) (ACIP, 1999). More recently a titre of 1:25 serum dilution has been suggested as evidence of exposure, as non-specific inhibition was not demonstrated at such dilutions (Jackson and Wunner, 2007). Our results support this suggestion as we observed many serum samples which neutralised both MLV(rabies) and MLV(VSV) at serum dilutions of 1:10 and 1:20. The selection of appropriate cut-off titres for neutralisation assays is critical for result interpretation, choosing a titre that is too low will inevitably cause false positive results and distort the interpretation of genuine serological data.

A titre of 0.5 IU/ml is recognised globally as the accepted rabies VNAb titre considered adequate to provide protection in humans post vaccination (ACIP, 1999). However, this threshold has since been extrapolated to other situations, and other species, in which such a titre may not apply (Moore and Hanlon, 2010). There is no accepted threshold, either measured as a serum dilution or in

IU/ml, for evidence of natural exposure in humans or animals. A wide range of thresholds have been used in the literature, with threshold levels as low as 0.06 IU/ml, to a much more conservative cut-off titre of 0.8 IU/ml (Ellison et al., 2014, Reynes et al., 2004). The complexity of the issue of threshold selection can be demonstrated using an example from Moore and Hanlon (2010) who analysed rabies VNAb titres recorded in vaccinated raccoons using the RFFIT. Analysing results using a 0.1 IU/ml threshold provided a sensitivity of 86% and a specificity of 83%, whereas the sensitivity decreased to 74% when a cut-off of 0.5 IU/ml was applied, however test specificity increased to 98% (Moore and Hanlon, 2010). Prior to determining a cut-off titre for use in a serosurvey, consideration must be given to the particular study group and the experimental questions being explored and a threshold that is 'fit for purpose' should be employed.

For rabies VNAb titres to be expressed in IU/ml, the OIE reference standard serum is required, however we were unable to acquire enough of this serum for our assays. Instead we employed a cut-off titre of 40, which was considered adequate for the research questions being investigated. This threshold is slightly higher than the suggested 1:25 dilution, however by selecting a slightly higher threshold we are increasing the specificity of the assay and in turn, as suggested by Reynes et al. (2004), these samples should be considered highly indicative of rabies-specific VNAb.

Evidence of potential rabies exposure in our longitudinal Kenyan dog cohort was demonstrated at the final sampling time point, at which high rabies VNAb titres were detected. A high proportion of dogs demonstrated an apparent anamnestic response to rabies virus, which was not observed at any other point throughout the 14-month study. Geographical clustering of these dogs provided further evidence of a common source of rabies exposure. No rabies cases were officially reported, or noted by the original authors of the study, at this time point or in the following months. Given that over half of the study dogs demonstrated detectable rabies VNAb at the beginning of the study, with no history of prior vaccination, and that evidence of rabies transmission during the study period was detected, it is possible that non-symptomatic rabies infection occurs more often than we realise in rabies endemic settings. Improved understanding of factors that differentiate fatal from non-fatal rabies exposures in nature will

enhance our understanding of the natural course of rabies infection and in turn contribute to the eradication of canine rabies.

7.4 Rift Valley fever virus serology

Many gaps still exist in our understanding of the complex ecology of RVFV. The virus infects a wide range of host species, infections in some of which appear to be asymptomatic. Additionally the periodic nature of RVFV outbreaks presents numerous questions regarding the maintenance of the virus during inter-epidemic periods (IEP).

In this thesis I present evidence of RVFV exposure in a range of wildlife and domestic livestock species. We present data regarding RVFV seroprevalence in lions in the SNP, further supporting the few previous studies available on RVFV infection in this species. Additionally, we present evidence of widespread infection in African buffalo in Northern Tanzania in addition to demonstrating RVF VNAbs in gazelle from this region. We demonstrate novel serological evidence of RVFV infection in Grant's gazelle, a species in which seroprevalence has not been previously documented in the literature. In both African buffalo and gazelle, the highest seroprevalence was observed in animals from the high risk region of Ngorongoro Conservation Area.

The age data associated with the serum samples tested in these studies are detailed and robust. As a result it is possible to confidently assert, from the presence of RVF VNAbs in animals that have only lived through IEP, that evidence of inter-epidemic transmission of RVFV has been identified. Low levels of inter-epidemic RVFV seroconversions were detected in all species tested in this study, namely lions, buffalo, gazelle, cattle, sheep and goats.

In Chapter 6, data generated from a large cohort of livestock enrolled in a longitudinal study is presented, which, to our knowledge, is the largest longitudinal study of RVFV seroprevalence performed to date. A number of interesting findings resulted from this study, one of which was the high proportion of animals under one year of age demonstrating RVF VNAbs, representing high IEP transmission of RVFV in these herds. Additionally, there was a wide variety in the seroprevalence of RVFV in different villages, with

seroprevalence ranging from 2-35%. However in contrast to the data generated in wild ruminants, the highest rates were not documented universally from villages in the highest risk areas.

Perhaps the most striking finding this study was the evidence of rapid waning of RVF VNAb in livestock. High levels of seroreversion in animals in the 6-8 month period of the study were observed. Levels of seroconversion and seroreversion were statistically associated with the village and herd to which the animals belonged, suggesting a geographical relationship in RVFV exposure. The data presented in this study suggest that high levels of seroreversion occur in villages where the least number of new RVFV infections occur, this pattern was particularly apparent in animals in the Loliondo district where the greatest differences in seroprevalence were observed. For example, a high proportion of seroreversions were observed in villages Nyamburi, Masusu and Kisangiro (50%, 63% and 82% respectively) and in these villages no seroconversions were detected during the study period. In contrast, Engaresero, Maaloni and Robanda had lower rates of seroreversion (20%, 33% and 30% respectively) and each had 3-5 animals within the village seroconverting during the study period.

Very little data is available in the literature regarding the duration of RVF VNAb following natural infection. As the date of exposure in these animals is unknown, we cannot comment on the duration of VNAb after natural infection. However, we describe low-level circulation of RVFV in this cohort, and in wildlife, during IEP, suggesting that RVFV is present outwith reported outbreaks. The mode of transmission may be either via mosquitoes or directly from other infected animals, wild or domestic, and further investigations are required to answer some of these questions.

Outbreaks of RVFV occur periodically, often after periods of prolonged rainfall, however the risks associated with outbreaks are incredibly complex. Métras et al. (2015) described the risk factors associated with RVFV outbreaks and commented that to successfully predict future RVFV outbreaks knowledge of previously vaccinated individuals is required. In their models, previously infected regions were considered immune to future outbreaks under the assumption that 'natural RVFV infection causes life-long immunity' (Métras et al., 2015). The duration of protective antibodies elicited after natural infection requires further

study, to improve predictive models of RVFV outbreaks and inform contingency plans for response to RVFV infections. Further research has commenced in our institute to investigate the levels and duration of immunity acquired to natural infection in study cohorts in South Africa.

We hypothesise that the inter-epidemic transmission of RVFV may contribute to retaining herd immunity and therefore may provide protection against explosive RVFV outbreaks. If further studies elucidate an association between herd immunity and the occurrence of RVFV outbreaks, livestock herds may be employed as sentinels to monitor RVFV circulation and determine when critical levels of immunity may diminish allowing devastating outbreaks to occur.

In summary, in this thesis I have optimised neutralisation assays to two important zoonotic diseases, the understanding of which can be improved by increasing the availability, and reliability, of serological assays. Preliminary seroprevalence data generated using these assays, from a wide range of ecological settings, are presented in Chapters 4 and 6. The data presented support previous serological studies in which data were generated using conventional antibody detection methods, providing evidence that these novel assays can provide robust serological data to further improve our understanding of rabies and RVFV.

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